Etude des causes génétiques de dérégulation du métabolisme de la vitamine D

Arnaud Molin

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Arnaud Molin. Etude des causes génétiques de dérégulation du métabolisme de la vitamine D. Médecine humaine et pathologie. Normandie Université, 2019. Français. NNT : 2019NORMC405 . tel-02349147

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THÈSE

Pour obtenir le diplôme de doctorat

Spécialité ASPECTS MOLECULAIRES ET CELLULAIRES DE LA BIOLOGIE

Préparée au sein de l'Université de Caen Normandie

Etude des causes génétiques de dérégulation du métabolisme de la vitamine D

Présentée et soutenue par
Arnaud MOLIN

Thèse soutenue publiquement le 09/10/2019 devant le jury composé de		
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Thèse dirigée par HERVE MITTRE et MARIE-LAURE KOTTLER, Oestrogènes, reproduction, cancer (Caen)

Remerciements

A **Madame le Professeur Véronique David**,
A **Monsieur le Professeur Pascal Houillier**,
Merci pour l'honneur que vous me faites de juger ce travail en tant que rapporteurs, d'avoir immédiatement accepté cette charge.

A **Madame le Docteur Caroline Silve**,
Merci pour l'honneur que vous me faites de juger ce travail.
Merci également pour vos encouragements à m'impliquer dans le groupe de travail « Outils du diagnostic » de la filière OSCAR.

A **Madame le Professeur Justine Bacchetta**,
Merci pour l'honneur que tu me fais de juger ce travail.
Merci pour notre précieuse collaboration Lyon-Caen pour l'étude des anomalies génétiques du métabolisme phosphocalcique, pour tes conseils et encouragements.

A **Monsieur le Professeur Christian Marcelli**,
Merci pour l'honneur que vous me faites de juger ce travail, ce que vous avez immédiatement accepté avec enthousiasme.

A **Madame le Professeur Marie-Laure Kottler**,
Merci pour l'honneur que vous me faites de juger ce travail.
Merci de m'avoir initié au métabolisme phosphocalcique, pour les longues heures passées à corriger les manuscrits, pour votre pugnacité, pour m'avoir appris et encourager à <u>oser</u>.

A **Monsieur le Docteur Hervé Mittre**,
Merci pour l'honneur que vous me faites de juger ce travail.
Merci pour vos encouragements constants et vos conseils depuis toutes ces années.

Aux patients et aux médecins dont le travail a contribué à l'élaboration de cette thèse, c'est à leur contact qu'on trouve la plus belle des motivations à continuer coute que coute à avancer,

A l'ensemble du personnel du service de Génétique du CHU de Caen pour leur bonne humeur et leur travail de qualité, qu'il ait été impliqué dans le travail de cette thèse de façon directe ou indirecte (en facilitant mon travail hospitalier) : techniciennes(iens) impliquées(és) et dévouées(és), secrétaires aux petits soins pour tenter d'organiser mes nombreuses activités pendant ces dernières années,

Merci aux groupes du midi pour cette ambiance de folie lors du déjeuner,

A mes collègues qui ont eu à supporter mon humeur changeante, espérons que cela change à l'avenir …

Aux membres des équipes de recherche OeReCa et BioTarGen, merci de m'avoir accueilli pour la réalisation de ce travail de thèse,

J'espère que les futurs projets apporteront satisfaction et épanouissement à tous.

Mention spéciale :

A Madame la Marquise de Ballandonne de Montérolier pour son humour piquant et son aide technique,

A Geneviève Abéguilé et Nadia Coudray qui ont accompagné mes premiers pas en biologie moléculaire,

A ma famille pour son indéfectible soutien,

A ma mère qui nous manque depuis bientôt 10 ans,

A mon père à qui je dois tant,

A mon frère et ma sœur et à leurs enfants et conjoints,

A mes amis,

Avant-propos

Depuis le séquençage de la totalité du génome humain en 2003, le diagnostic génétique de nombreuses maladies a considérablement évolué grâce aux avancées technologiques et une réduction constante de leur coût. Ces maladies sont improprement baptisées « maladies rares » car elles concernent en réalité un grand nombre de patients (1/2000 personnes en France) et sont en fait largement sous-diagnostiquées. Cela a justifié un soutien récent du Ministère de la Santé et la volonté de structurer l'offre de soin en labellisant des centres de référence puis des filières maladies rares.

Au sein du laboratoire de Génétique du CHU de Caen attaché au Centre de Référence Constitutif Normand des Maladies Rares du Métabolisme du Calcium et du Phosphate et adossé à l'EA7450 BIOTARGEN, nous nous sommes intéressés aux anomalies du métabolisme de la vitamine D et avons pu bénéficier d'un recrutement national.

Notre objectif au cours de ce travail de thèse a été de développer de nouvelles stratégies diagnostiques qui ont permis d'améliorer la prise en charge des patients, de disséquer les mécanismes physiopathologiques de ces maladies, afin de proposer aux patients des prises en charges et thérapeutiques adaptées dans un concept de médecine personnalisée.

Le mémoire que je présente s'appuie sur trois publications majeures et est organisé de la façon suivante :

- une vaste introduction dans laquelle je rappellerai la synthèse de la vitamine D, son métabolisme, son mode d'action et les maladies associées aux anomalies du métabolisme de la vitamine D,
- les différentes publications et les résultats obtenus,
- enfin, un dernier chapitre dédié à la discussion qui me permettra de proposer des hypothèses physiopathologiques dont la confirmation sera l'objet de mon travail ultérieur.

RESUME

La vitamine D (D_3 ou cholécalciférol du règne animal et D_2 ou ergostérol du règne végétal) est une hormone pléiotrope qui possède de nombreux effets biologiques incluant la régulation du métabolisme du calcium et du phosphate. Chez l'Homme, ce composé est synthétisé au niveau cutané sous forme inactive. On décrit ainsi le métabolisme de la vitamine D qui conduit à la production de métabolites actifs (par les vitamine D 25- et 1α-hydroxylases codées par les gènes *CYP2R1* et *CYP27B1*) et à leur dégradation par la vitamine D 24-hydroxylase (gène *CYP24A1*). L'expression des vitamine 1α- et 24-hydroxylases est finement et inversement régulée afin de maintenir l'homéostasie phosphocalcique, grâce à plusieurs boucles de rétrocontrôle impliquant entre autres la forme 1,25-dihydroxylée de la vitamine D et son récepteur VDR, la calcémie et la parathormone, la phosphatémie et le FGF23. La carence en vitamine D et les défauts de son activation sont associés à un phénotype de rachitisme, tandis que les excès en vitamine D sont associés à un phénotype d'hypercalcémie-hypercalciurie par intoxication (surdosage) ou hypersensibilité à la vitamine D (excès d'activation ou défaut de dégradation).

L'objectif de ce travail de thèse est d'identifier des causes génétiques de dérégulation du métabolisme de la vitamine D et de préciser leurs mécanismes physiopathologiques par une description précise du phénotype associé. Pour ce faire, nous avons utilisé de façon conjointe les outils de la génétique (séquençage nouvelle génération et Sanger) et de la biochimie (dosage des métabolites) dans une cohorte de patients recrutés grâce au centre de référence maladies rares du métabolisme du calcium et du phosphate.

Ce travail a permis de préciser le rôle de deux gènes dans les maladies liées à la dérégulation métabolisme de la vitamine D, *CYP2R1* et *CYP24A1*, par la mise en évidence de mutations perte de fonction chez des patients avec un phénotype de rachitisme à 25-hydroxyvitamine D basse et d'hypersensibilité à la vitamine D respectivement. Notre étude a permis aussi de préciser le phénotype de ces affections. Dans la cohorte des patients étudiés, l'identification de mutations de gènes impactant le métabolisme du phosphate (*SLC34A1* et *SLC34A3*), souligne l'intérêt de l'étude des facteurs régulateurs des activités vitamine D 1α- et 24-hydroxylases.
Aucune variation significative dans les régions promotrices proximales de *CYP27B1* et *CYP24A1* n'a été identifiée. Le peu de connaissances sur l'ensemble des éléments régulateurs chez l'Homme n'a pas permis d'approfondir notre étude. L'identification et l'étude de ces

éléments régulateurs distaux permettra de déterminer leur implication dans les maladies rares du métabolisme de la vitamine D.

ABSTRACT

The vitamin D (D_3 or cholecalciferol from animal kingdom and D_2 or ergosterol from plan kingdom) is a pleiotropic hormone who has numerous biological effects including the regulation of calcium and phosphate metabolism. In humans, this compound is synthetized in skin in an inactive form. Thus, we call vitamin D metabolism the biological process which leads to the production of active metabolites (by enzymes 25- and 1α-hydroxylases encoded by *CYP2R1* and *CYP27B1* genes) and its degradation by vitamin D 24-hydroxylase (gene *CYP24A1*). The expression of 1α- and 24-hydroxylases is tightly and inversely regulated to maintain calcium and phosphate homeostasis, thanks to several feedback loops including 1,25-dihydroxyvitamin D and its receptor VDR, serum calcium and parathormone, serum phosphate and FGF23. Vitamin D deficiency and vitamin D activation deficiency are associated with rickets, while vitamin D excess are associated with hypercalcemia-hypercalciuria due to vitamin D intoxication (overdose) or hypersensitivity to vitamin D (activation excess or degradation deficiency).

Our aim is to identify genetic causes of vitamin D metabolism deregulation and to specify pathophysiologic mechanisms describing phenotype. Thus, we jointly used the tools of genetics (next-generation and Sanger sequencing) and biochemistry (vitamin D metabolites assay) in a cohort of human patients ascertained thanks to the national center for rare diseases of calcium and phosphate metabolism.

This work allowed us to specify the role of two genes in diseases of vitamin D metabolism, *CYP2R1* and *CYP24A1*, showing loss of function mutations in patients with rickets and low 25-hydroxyvitamin D and hypersensitivity to vitamin D, respectively. Our study brought new phenotypic elements in these affections. In our cohort of patients, the identification of mutations leading to phosphate deregulation (in *SLC34A1* and *SLC34A3*) highlights the putative role of regulators of vitamin D 1α- and 24-hydroxylases activities in pathophysiology.
No significant variation have been identified in the proximal promoting regions of *CYP27B1* and *CYP24A1*. We could not go further considering the lack of knowledge in regulating regions and factors in humans. Identifying distal regulators will allow to study their implication in rare diseases of vitamin D metabolism.

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A. **Molin A**, Baudoin R, Kaufmann M, Souberbielle JC, Ryckewaert A, Vantyghem MC, Eckart P, Bacchetta J, Deschenes G, Kesler-Roussey G, Coudray N, Richard N, Wraich M, Bonafiglia Q, Tiulpakov A, Jones G, Kottler ML. *CYP24A1* Mutations in a Cohort of Hypercalcemic Patients: Evidence for a Recessive Trait. *J Clin Endocrinol Metab.* 2015 Oct;100(10):E1343-52 ……………………………………………………………63

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c. Kaufmann M, Morse N, Molloy BJ, Cooper DP, Schlingmann KP, **Molin A**, Kottler ML, Gallagher JC, Armas L, Jones G. Improved Screening Test for Idiopathic Infantile Hypercalcemia Confirms Residual Levels of Serum 24,25-$(OH)_2D3$ in Affected Patients. *J Bone Miner Res.* 2017 Jul;32(7):1589-1596

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e. Bertholet-Thomas A, Tram N, Dubourg L, Lemoine S, **Molin A**, Bacchetta J. Fluconazole as a New Therapeutic Tool to Manage Patients With NPTIIc (SLC34A3) Mutation: A Case Report. *Am J Kidney Dis.* 2019 Feb 11. pii: S0272-6386(19)30006-X.

Liste des abréviations

1α-OH D_3 : alfacalcidol

1,25-$(OH)_2D$: 1,25-dihydroxyvitamine D (calcitriol)

24,25-$(OH)_2D$: 24,25-dihydroxyvitamine D

25-OH-D : 25-hydroxyvitamine D (calcifédiol)

3C : *Chromosome Conformation Capture*

7-DHC : 7-déhydrocholestérol

AMPc : AMP cyclique, Adénosine MonoPhosphate cyclique

BAC : chromosome artificiel de bactérie

ChIP-chip : immunoprecipitation de la chromatine couplée à l'hybridation génomique sur puce à ADN

ChIP-seq : immunoprecipitation de la chromatine couplée au séquençage

CoA : coenzyme A

CYP450 : enzyme associée aux cytochromes P450

CREB : *cAMP Response Element-binding protein*

DBD : *DNA binding domain*, domaine de liaison à l'ADN

DBP : *D binding protein*, protéine de transport de la vitamine D

FGF23 : *fibroblast growth factor 23*

GRIO : Groupe de Recherche et d'Information sur les Ostéoporoses

GWAS : *Genome Wide Association Study*

HMG : hydroxyméthylglutarate

HVD : hypersensibilité à la vitamin D

LC-MS/MS : chromatographie liquide couplée à la spectrométrie de masse en tandem

MPS : séquençage multiple en parallèle ou *multiple parallel sequencing*

$NADP^+$/NADPH : Nicotinamide adénine dinucléotide phosphate (oxydé/réduit)

NGS : séquençage nouvelle génération ou *next generation sequencing*

nVDRE : *negative vitamin D response element* (répresseur)

PAL : phosphatase alcaline

PTH : parathormone

PTHrp : *PTH related peptide*

RXR : récepteur à l'acide rétinoïque (vitamine A)

SNP : *single nucleotide polymorphism*, polymorphisme d'un seul nucléotide

TAD : *topologically associated domain*, domaine topologiquement associé

TSS : *transcription start site*, site d'initiation de la transcription

UI : unité internationale

UVB : rayonnements ultraviolets B

VDDR : *vitamin D dependent rickets*, rachitisme vitaminorésistant

VDR : *vitamin D receptor*

VDRE : *vitamin D response rlement*

VSE : *vitamin D stimulating element*

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INTRODUCTION

La vitamine D

Historique

L'histoire de la vitamine D est indissociable de celle du rachitisme, maladie connue depuis l'Antiquité bien qu'elle n'ait été décrite avec précision pour la première fois qu'au dix-septième siècle par les Anglais Daniel Whistler (Leyden, 1645) et Francis Glisson (Londres, 1650). Elle a constitué un fléau dans les populations d'enfants européens pendant des siècles, surtout à partir de la révolution industrielle dans les régions à faible ensoleillement (Royaume Uni). Sa prévalence au vingtième siècle était telle, que les lésions osseuses caractéristiques du rachitisme (déformations, élargissements métaphysaires) étaient présentes chez tous les enfants âgés de moins de 18 mois qui mouraient lors des mois d'hiver en Europe du Nord[1]. Pour les médecins de l'époque, il s'agissait d'une maladie du squelette, non héréditaire et non contagieuse, qui atteignait préférentiellement les enfants de famille aisée. De nombreuses hypothèses ont été avancées pour l'expliquer, depuis la théorie des humeurs avancée par Glisson à la mauvaise hygiène de vie ou à la pollution.

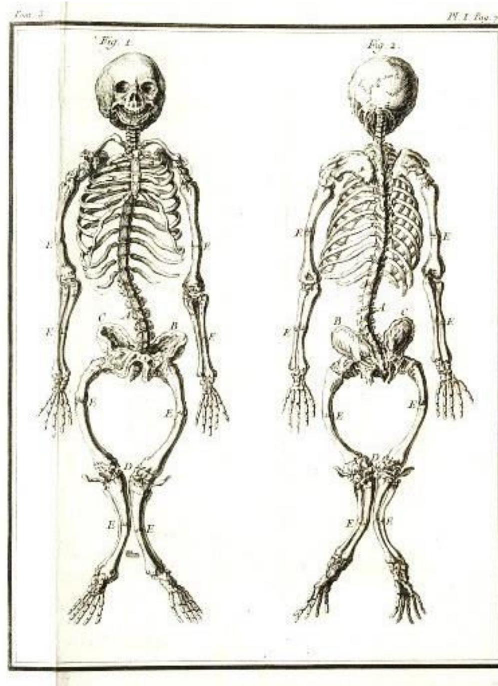

Figure 1 : Squelette d'un enfant rachitique, gravure, d'après Histoire naturelle (1789)

Le premier traitement efficace du rachitisme vint de la pharmacopée traditionnelle du Nord de l'Europe : l'huile de foie de morue. Cette substance y était très employée comme fortifiant. Thomas Percival (1740-1804), médecin anglais du dix-huitième siècle, écrivit en 1782 la première publication sur les effets bénéfiques de cette huile dans les affections osseuses. Son utilisation pour lutter contre le rachitisme débuta lors du siècle suivant. En 1889, Blanc-Sutton guérit grâce à elle les lionceaux rachitiques du zoo de Londres, et avant lui en 1865, Armand Trousseau observait ses effets sur ses patients rachitiques. Ce dernier observa les mêmes effets bénéfiques produits par l'exposition au rayonnement solaire. En 1890, Palm compara la fréquence et la répartition géographique du rachitisme et émit l'hypothèse d'un manque d'exposition solaire à l'origine de la maladie.

L'origine carencielle de la maladie est prouvée en 1919 par l'anglais Edward Mellanby qui étudie des portées de chiots qu'il soumet à différents types de régime. Il conclut à la responsabilité d'une carence, soit en vitamine A, soit en une autre substance anti-rachitique inconnue, présente en grande quantité dans l'huile de foie de morue, et dans une moindre mesure, dans le beurre. L'existence de ce facteur anti-rachitique spécifique sera prouvée par les expériences de McCollum *et al.* dans les années 1920. En suivant l'ordre alphabétique, il sera baptisé « vitamine D » (la quatrième vitamine identifiée). Sa structure chimique sera caractérisée à la même époque, par Askew (vitamine D_2, 1931), Windaus et Boch (vitamine D_3, 1937). Ces derniers identifièrent également le 7-déhydrocholestérol, précurseur de la vitamine D, et mirent en évidence sa conversion en vitamine D_3 sous l'action des rayonnements ultraviolets, le lien entre rachitisme, vitamine D et exposition solaire étant ainsi établi. On note d'emblée le caractère ambigu associé à la définition de ce qu'est la vitamine D, molécule produite par l'organisme, ce qui s'oppose à la définition même du mot vitamine (substance organique active, sans valeur énergétique, indispensable en très faible quantité à la croissance et au bon fonctionnement de l'organisme qui ne peut en faire la synthèse et à qui elle est apportée par l'alimentation). Enfin, cette dernière découverte ouvrit également la voie à la production industrielle de vitamine D par irradiation de produits alimentaires. Ces travaux permirent une avancée majeure dans la lutte contre le rachitisme. L'utilisation de l'huile de foie de morue comme moyen de prévention se généralisa à partir des années 1930, puis l'enrichissement de divers produits alimentaires de consommation courante (lait et produits laitiers, céréales, farines et pain, …) et la supplémentation des populations de sujets à risque permit la quasi-éradication de cette maladie dans les pays développés. Le rachitisme demeure un problème de santé

publique dans certains pays en voie de développement et dans certaines populations à risque dans les pays occidentaux (sujets à peau noire dans des pays à faible taux d'ensoleillement, régimes alimentaires particuliers, …).

De rares cas de rachitismes résistants à la vitamine D, c'est-à-dire non corrigés par la prise d'huile de foie de morue ou de vitamine D purifiée, ni par l'exposition aux ultraviolets, ont par la suite attiré l'attention des médecins. Le premier a été décrit en 1937 par trois médecins américains, Albright, Butler et Bloomberg. Il s'agissait d'un garçon qui présentait un rachitisme clinique depuis l'âge de 15 mois. La prise d'huile de foie de morue n'avait pas guéri l'enfant, et il fut traité par de multiples ostéotomies. La prise massive de vitamine D permit une amélioration clinique transitoire au prix d'une importante hypercalcémie. Les progrès de l'hormonologie et de la biologie moléculaire à la fin du vingtième siècle ont permis d'expliquer ces rachitismes vitaminorésistants (VDDR), maladies génétiques, ce qui démontra le rôle important des facteurs génétiques dans la physiologie associée à la vitamine D.

Structure chimique

La vitamine D appartient au groupe des vitamines liposolubles (vitamines A, D, E et K). On en distingue une forme dérivée du cholestérol produit par les organismes du règne animal (poissons, mammifères, …) nommée vitamine D_3 ou cholécalciférol (*9,10-secocholesta-5,7,10(19)-trien-3-ol*), et une forme dérivée de l'ergostérol (*9,10-secoergosta-5,7,10(19),22-tetraen-3-o*l) produit par les plantes, les levures et les champignons, nommée vitamine D_2 ou ergocalciférol (**Figure 2**). Dans la suite de ce manuscrit, le terme vitamine D désigne ces deux composés de manière indifférenciée.

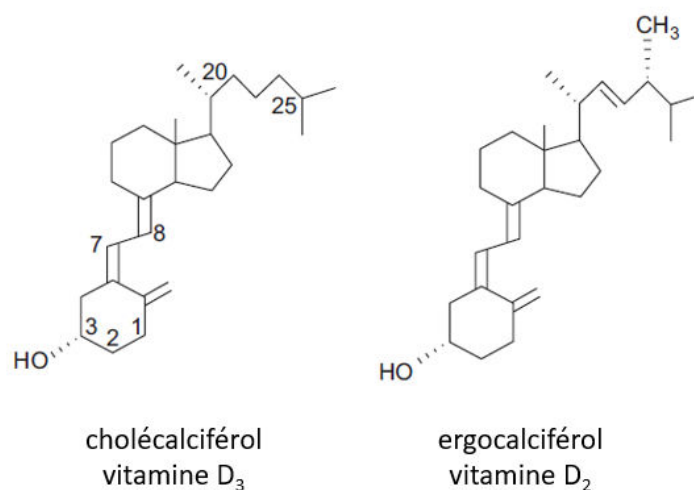

Figure 2 : Structure chimique des vitamines D_2 et D_3

Elle appartient au groupe des sécostérols, composés polycycliques complexes caractérisés par la présence d'un noyau stérane (ou cyclopentane-perhydrophénanthrène) fait de quatre cycles dont le second (cycle B) est ouvert (trois cycles de type cyclohexane A, B et C, accolés à un cycle de type cyclopentane, D) et d'une chaîne latérale hydrocarbonée. Le terme *seco* se rapporte au cycle B ouvert du noyau stérane. On distingue des formes *cis* ou *trans* selon la position du cycle A par rapport au plan formé par les cycles C et D, des conformations α-*chair* ou β-*chair* selon la position des groupements portés par les carbones 1, 3 et 19 par rapport à ce même plan. La conformation tridimensionnelle de la chaine latérale est également variable. Ainsi, la conformation tridimensionnelle de la vitamine D est variable, souple et dynamique, à l'origine de plus de 30 formes naturelles différentes de vitamine D.

Biosynthèse

Chez l'Homme, la production endogène de la vitamine D_3 a lieu au niveau cutané, dans la partie profonde de l'épiderme (couche des cellules de Malpighi et couche basale) riche en 7-déhydrocholestérol (7-DHC, localisé dans les membranes cellulaires). Cette photosynthèse de la vitamine D existe également chez de nombreuses espèces animales.

Le 7-DHC est un précurseur du cholestérol principalement synthétisé dans le cytoplasme des cellules du foie et de l'intestin. La condensation de molécules d'acétate (3) permet la synthèse d'hydroxyméthylglutarate (HMG) qui est réduit en mévalonate grâce à l'action de l'HMG-CoA réductase et du coenzyme A (CoA). Le mévalonate est ensuite décarboxylé en isoprénoïdes (molécules dérivées de l'isoprène ou 2-méthyl-1,3-butadiène, hydrocarbure ramifié à doubles liaisons : isopenténylet diméthylallyl, dits activés par liaison au pyrophosphate) dont la condensation permet la synthèse de squalènes (hydrocarbures polyisopréniques résultant de la polymérisation de 6 unités isoprènes). Enfin, la squalène cyclase crée les cycles composant le noyau stérane à partir des insaturations présentes dans la molécule de squalène, donnant lieu à la synthèse de lanostérol qui sera par la suite converti en 7-DHC puis en cholestérol. Une synthèse à partir de molécules d'acide gras à chaine courte ou de leucine est également possible.

Sous l'action du rayonnement ultraviolet B (UVB) (longueur d'onde de 290 à 315 nm), le cycle B du noyau stérane s'ouvre pour former la pré-vitamine D_3 (**Figure 3**). Il s'agit d'une molécule thermodynamiquement instable, convertie en vitamine D_3 ou cholécalciférol. Environ 50 % de la pré-vitamine D_3 peut être convertie en cholécalciférol en 2 heures. Deux isomères

biologiquement inactifs sont également issus de la pré-vitamine D_3, surtout en cas d’exposition solaire prolongée : le lumistérol et le tachystérol.

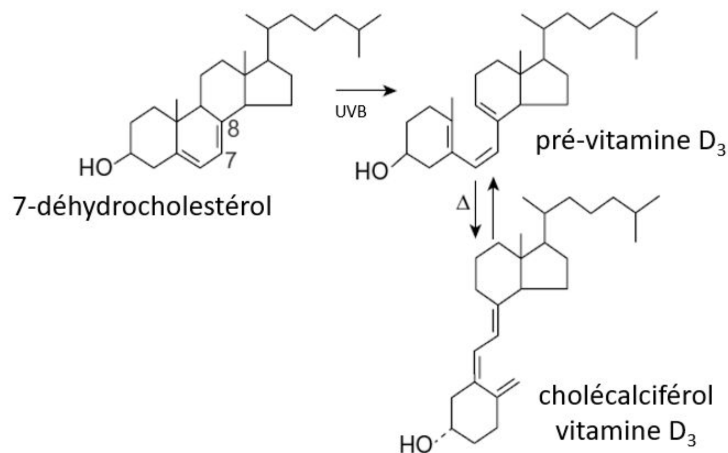

Figure 3 : Biosynthèse de la vitamine D : conversion du 7-déhydrocholestérol en pré-vitamine D instable sous l’action des rayons UVB, puis conversion de la pré-vitamine D en cholécalciférol

Environ 80 à 90 % des besoins en vitamine D d’un adulte proviennent de la production endogène, les 10 à 20 % restant étant apportés par l’alimentation. La vitamine D n’est donc pas une vitamine au sens strict du terme (composé essentiel, qui ne peut être produit par l’organisme). La photosynthèse de la vitamine D_3 dépend directement de l’exposition solaire, à la fois de sa durée, de sa qualité (longueurs d’onde appropriées) et de son intensité, d’où l’existence de variations saisonnières et géographiques (**Figure 4**).

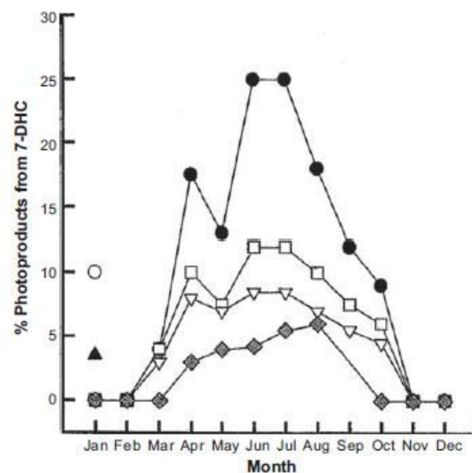

Figure 4 : Variation annuelle de la production de pré-vitamine D_3
Mesures réalisées à Boston (triangle et carré blanc après 1h et 3h d'exposition solaire ; rond noir : mesure incluant la production de tachystérol et lumystérol), Edmonton (carré noir), Los Angeles (triangle noir) et Puerto Rico (rond blanc). D'après Webb AR, Kline L, Holick MF *J Clin Endocrinol Metab* 1988;67:373–8. On observe une augmentation de la production de pré-vitamine D_3 à partir du 7-DHC durant les mois à plus fort ensoleillement.

Ainsi, la forte prévalence de la carence en vitamine D dans les pays à faible ensoleillement s'explique facilement, de même que la forte susceptibilité à la carence vitaminique des populations à peau noire vivant dans ces mêmes pays (la forte quantité de mélanine absorbe les UVB, inhibant la conversion du 7-DHC), en cas de port de vêtements couvrants la totalité du corps (facteur de risque culturel). Enfin, l'appauvrissement de la peau du sujet âgé en 7-DHC explique en partie l'augmentation de la fréquence de la carence vitaminique avec l'âge[2].
Après conversion du 7-DHC en cholécalciférol, la vitamine D_3 quitte la membrane plasmique pour entrer dans le compartiment extra-cellulaire et rejoindre la circulation sanguine par l'intermédiaire des capillaires du derme. Elle y est captée par sa principale protéine de transport, la DBP (*D Binding Protein*) ou, dans une moindre mesure, par d'autres protéines de transport (albumine, lipoprotéines), véhiculée jusqu'aux tissus et convertie en différents métabolites, notamment en 25-hydroxyvitamine D (25-OH-D, forme dite de stockage) et en 1,25-dihydroxyvitamine D (1,25-$(OH)_2$D, forme dite active). Au niveau rénal, la 25-OH-D est filtrée dans le glomérule, puis réabsorbée dans le tubule contourné proximal par endocytose après fixation de la DBP à la mégaline, protéine transmembranaire agissant comme un récepteur multiligand[3], mais aussi à d'autres protéines récepteurs (cubuline[4], disabled-2[5]). Ainsi, le

modèle murin invalidé pour la mégaline est incapable de réabsorber la 25-OH-D, et présente en conséquence un phénotype de rachitisme et un défaut de synthèse de la 1,25-$(OH)_2D$ [6].

Le récepteur de la vitamine D

Le récepteur de la vitamine D est codé par le gène *VDR* (12q13.11), exprimé dans l'ensemble des tissus cibles de la vitamine D. A l'échelle cellulaire, le *VDR* peut être localisé dans le noyau[7–9] et dans le cytosol dans des domaines associés aux radeaux lipidiques de type cavéole lipidique[10,11]. Il s'agit d'une protéine de 427 acides aminés dont la séquence est fortement conservée ; appartenant à la famille des récepteurs nucléaires aux hormones stéroïdes. Ce récepteur possède des fonctions de facteur de transcription (il lie l'ADN pour favoriser ou inhiber l'expression de gènes cibles)[12,13]. L'étude de sa structure révèle la présence de différents domaines fonctionnels : un domaine de liaison à l'ADN avec doigts à Zinc (DNA Binding Domain, DBD), un domaine de reconnaissance du ligand et un domaine d'interaction avec d'autres facteurs de transcription (co-represseurs ou co-activateurs) (**Figure 6**).

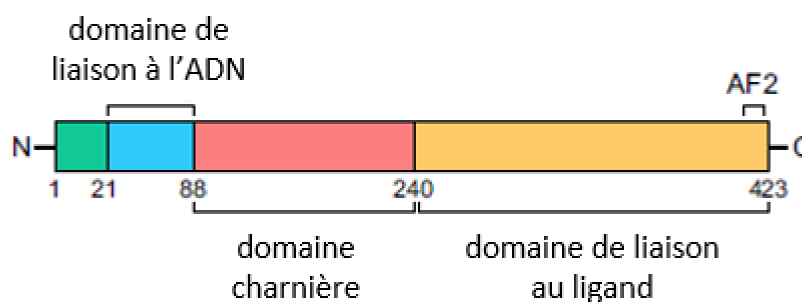

Figure 5 : Représentation schématique du récepteur de la vitamine D
Le récepteur de la vitamine D comprend (de l'extrémité N-terminale vers l'extrémité C-terminale) un domaine de liaison à l'ADN, un domaine charnière conférant sa flexibilité à la molécule, un domaine de liaison au ligand (vitamine D, hétérodimérisation avec le récepteur au rétinoïde RXR, interaction avec la machinerie transcriptionnelle) et enfin un domaine transactivateur AF-2.

Mécanismes d'action de la vitamine D

On distingue communément deux mécanismes d'action selon le temps nécessaire à la mesure d'une réponse cellulaire après traitement par la 1,25-$(OH)_2D$[20] : une voie lente et une voie rapide. Selon sa conformation tridimensionnelle, la 1,25-$(OH)_2D$ active l'une ou l'autre de ces voies.

La voie lente dite nucléaire ou génomique est liée à la translocation du récepteur dans le noyau pour moduler l'expression des gènes (transcription puis traduction). La voie rapide (effets observés quelques secondes à quelques minutes après traitement)[21] est dite extranucléaire ou non-génomique liée aux interactions de la fraction cytosolique du récepteur avec d'autres molécules intracellulaires. Ces deux voies fonctionnent de concert afin de réguler la physiologie cellulaire.

La voie génomique est la voie la plus connue, classiquement décrite pour expliquer la régulation de l'expression génique par la vitamine D (exemple de l'induction de l'expression de *CYP24A1*). Après fixation de la vitamine D au VDR, celui-ci s'hétérodimérise avec le récepteur RXR (*Retinoid X Receptor*, de type α, β ou γ)[22] (on ignore si l'hétérodimérisation a lieu dans le cytosol ou le noyau[23,24]), liaison de l'hétérodimère à l'ADN au niveau de sites de reconnaissance spécifiques nommés *Vitamin D Responsive DNA Element* (VDRE) (**Figure 7**). La liaison du complexe 1,25-$(OH)_2$D-VDR-RXR au VDRE permet le recrutement de complexes protéiques impliqués dans le remodelage chromatinien (complexe SWI/SNF[25]), la modification des histones (acétyltransférases, méthyltransférases, déacétylases et déméthylases)[26,27], le recrutement de facteurs de transcription[28], la liaison à l'ADN de l'ARN polymérase II et l'initiation de la transcription (complexe Médiateur)[29].

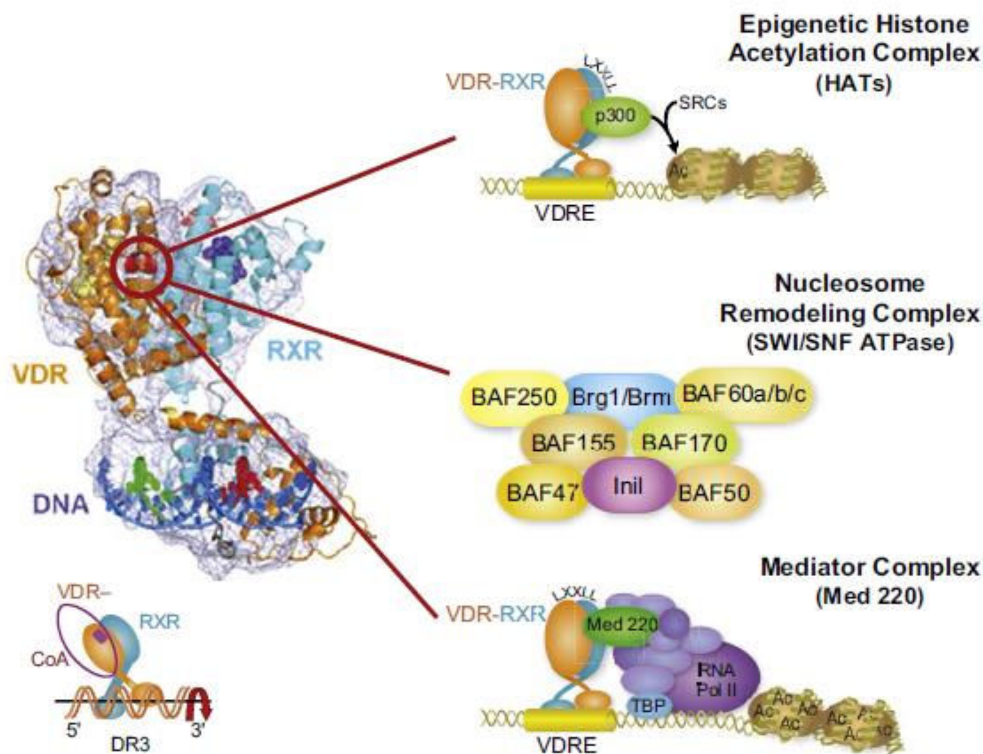

Figure 6 : Voie d'action génomique de la vitamine D

La fixation de l'hétérodimère VDR-RXR au VDRE permet le recrutement de complexes multiprotéiques impliqués dans le remodelage de la chromatine, les modifications des histones (exemple des coactivateurs (CoA) des récepteurs aux stéroïdes (SRC) et de l'histone acétyltransférase p300), le remodelage du nucléosome (complexe SWI/SNF ATPase), l'initiation de la transcription (complexe Médiateur Med220 et ARN polymérase II). L'ensemble permet d'ouvrir la chromatine et d'initier la transcription.

Des études aux loci de gènes fortement induits par la vitamine D (ostéocalcine, ostéoprotégérine, vitamine D 24-hydroxylase) ont initialement permis de préciser la séquence des VDRE activateurs : une séquence de 15 pb constituée d'une répétition directe de deux hexanucléotides AGGTCA séparées par 3 pb variables (*DR3 element*) (AGGTCAxxgAGGTCA) et localisée à environ 1 kb en amont des gènes cibles[30–36]. Plus récemment, des études combinant séquençage massif en parallèle (MPS) et immunoprécipitation de la chromatine (ChIP-chip ou ChIP-seq) ont permis d'identifier un grand nombre de séquences ADN capable de lier le VDR seul, et un nombre encore plus élevé de loci capables de lier le complexe 1,25-$(OH)_2$D-VDR-RXR[37,38]. Ces sites de fixation peuvent être localisés dans des régions introniques ou intergéniques, parfois à des centaines de kb en amont ou en aval du site d'initiation de la transcription ; ils sont le plus souvent multiples et

fréquemment regroupés en cluster[39]. L'ensemble de ces sites est spécifique d'un type cellulaire donné et est nommé cistrome. Le cistrome varie au cours du développement (différenciation cellulaire), en cas de processus pathologique[40]. La séquence du (des) VDRE répresseur(s) (nVDRE) n'est pas connue.

La voie non-génomique est notamment impliquée dans la régulation d'activité de canaux ioniques, de kinases, phosphatases et phospholipases, et peut donc indirectement également aboutir à une modification d'expression génique (transcription ou traduction). L'utilisation d'analogues synthétiques de la vitamine D constitue un outil d'intérêt pour l'étude de cette voie, par la comparaison d'analogues de la vitamine D dépourvus d'activité agoniste génomique ou non-génomique.

Analogues synthétiques

De nombreux analogues de vitamine D synthétiques (**Figure 5**) sont à ce jour décrits, capables de lier le récepteur de la vitamine D pour en reproduire tout ou partie des effets (agonistes) ou pour s'opposer aux effets de la vitamine D (antagonistes). Certains d'entre eux ne diffèrent de la 1,25-$(OH)_2D$ que par leur structure tridimensionnelle, mais présentent des différences en termes d'affinité et d'activité. Ces analogues dont la structure tridimensionnelle est fixe (contrairement à celle de la vitamine D) permettent d'étudier les fonctions associées aux différentes conformations de vitamine D.

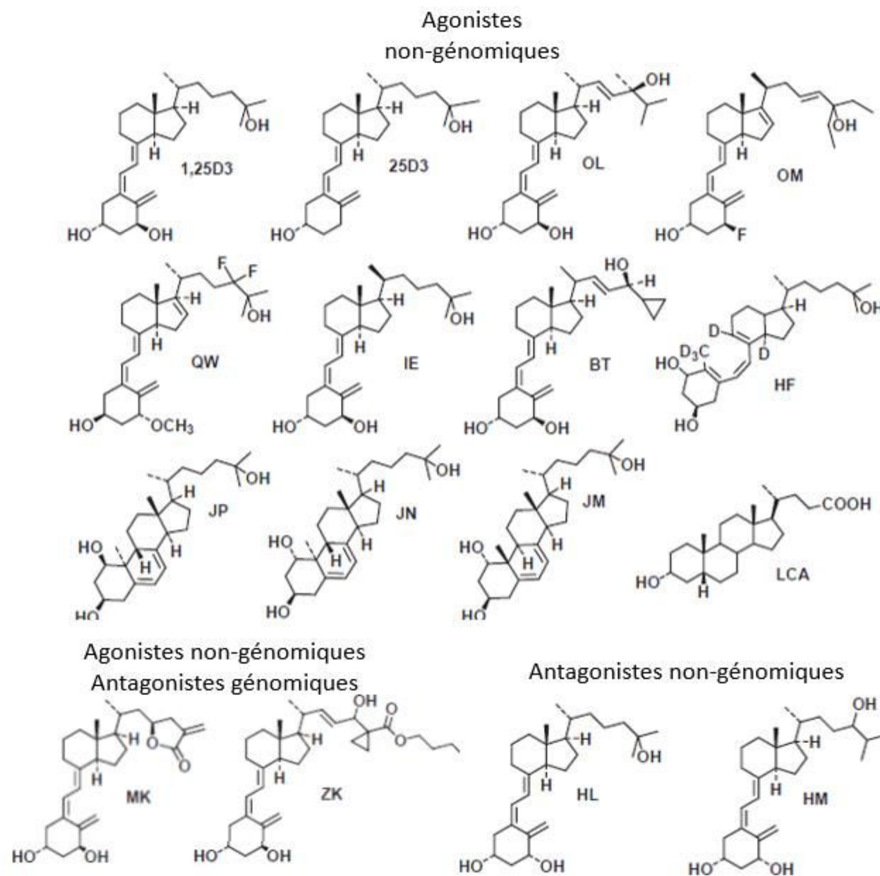

Figure 7 : Molécules analogues de la vitamine D capables de se lier au VDR
Les analogues synthétiques sont désignés par un code formé de deux lettres (exemple : ZK). Tous ces analogues ont des propriétés agonistes non-génomiques sauf HL et HM. Tous ces analogues ont des propriétés agonistes génomiques sauf MK et ZK.

Fonctions biologiques

Actions de la vitamine D dans le métabolisme phosphocalcique

La vitamine D apparait comme une hormone clé de la régulation de l'homéostasie du calcium et du phosphate grâce à une action au niveau intestinal, osseux et rénal. Au niveau intestinal, la 1,25-$(OH)_2$D favorise l'expression de gènes impliqués dans le transport actif de calcium depuis la lumière intestinale (calbindine D9K codée par le gène *S100G*, transporteurs de calcium codés par les gènes *SLC8A2* (NCX1, échangeur sodium-calcium 1), *TRPV6* ou *ATP2B1*)[41]. Au niveau osseux, elle favorise la mobilisation du calcium et du phosphate en stimulant l'activité et la différenciation ostéoclastique[42–44] en stimulant l'activation de la voie NF-κB (expression du

récepteur au TNFα, de RANKL), de facteurs régulant la minéralisation osseuse (ostéopontine, matrix-gla protéine, ectonucléotide pyrophosphatase/phosphodiestérase, transporteur membranaire de pyrophosphate, phosphatase alcaline)[45], mais aussi du FGF23, hormone régulatrice du métabolisme du phosphate produite par les ostéocytes[46]. Enfin, au niveau rénal, elle favorise la réabsorption du calcium filtré en stimulant l'expression de transporteurs au niveau du tubule contourné distal (calbindines, pompes calciques, canaux épithéliaux, échangeur sodium-calcium)[47,48], du récepteur de la PTH[49]. Néanmoins, la majeure partie de la réabsorption du calcium filtré a lieu dans le tubule contourné proximal, par un mécanisme paracellulaire dépendant de la concentration urinaire en sodium et indépendant de la vitamine D.

Actions en dehors du métabolisme phosphocalcique

La mise en évidence d'une expression du récepteur de la vitamine D en dehors des tissus impliqués dans le contrôle du métabolisme du calcium et du phosphate (kératinocytes, lymphocytes, cellules pancréatiques, …) a suggéré l'implication de la vitamine D dans d'autres processus biologiques[50,51], notamment la régulation de la prolifération (progression du cycle cellulaire, apoptose) et de la différentiation cellulaire. Ces mécanismes sont étudiés par exposition *in vitro* de différents types cellulaires à de la vitamine D (1,25-$(OH)_2D$) ou à des analogues synthétiques.
Sur le plan du métabolisme cellulaire, la vitamine D s'oppose à la progression du cycle cellulaire en favorisant des arrêts, principalement lors de la transition entre les phases G0 et G1 (une promotion des arrêts en phase G1 ou lors de la transition G2-M a également été proposée[52,53]), par une régulation positive de la production d'inhibiteurs des kinases cyclines dépendantes[54–58] ou par une régulation négative de la production de cyclines[59–61]. Elle favorise également l'apoptose, en modulant l'expression de facteurs pro-apoptotiques (voie des caspases[62,63], famille des protéines BCL2[64–66]) et anti-apoptotiques (IGF1[67]) dans de nombreux types cellulaires, normaux comme tumoraux. Des expériences *in vitro* de traitement de lignées cancéreuses par de la vitamine D ou l'un de ses analogues suggèrent des effets positifs sur la différentiation par la mise en évidence de modifications du phénotype cellulaire (expression de marqueurs épithéliaux dans des cellules de carcinome épidermoïde SCC-25[68], de marqueurs myoépithiéliaux dans des cellules de cancer du sein[69], formation de microvillosités dans des entérocytes Caco-2[70]).

Métabolisme de la vitamine D

La vitamine D formée au niveau cutané n'est pas biologiquement active car son affinité pour son récepteur est faible. Elle subit une activation séquentielle en deux étapes pour augmenter massivement cette affinité et exercer ses nombreuses fonctions biologiques (**Figure 8**). Par la suite, elle est dégradée afin de réguler ces effets.

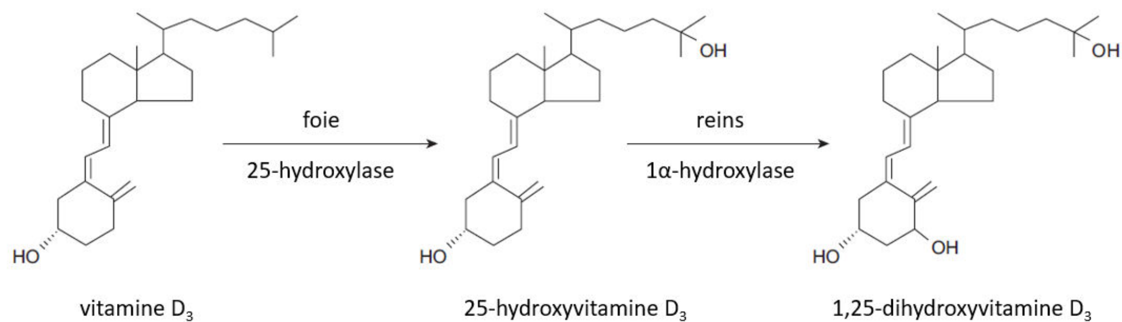

Figure 8 : Activation séquentielle de la vitamine D_3

Ces fonctions d'activation et de dégradation constituent le métabolisme de la vitamine D. Elles sont catalysées par des enzymes associées aux cytochromes P450 (CYP450) capables d'hydroxyler la vitamine D en des points précis de sa structure, sur les carbones en position 1α, 25 et 24 de sa chaine latérale notamment. L'oxydation sur les carbones 25 et 1α n'altère pas la flexibilité de conformation de la vitamine D, contrairement à celle sur les carbones 23 et 24[71].

Généralités sur les enzymes associées aux cytochromes P450

Les CYP450 sont des enzymes qui réalisent l'oxydation (activité monooxygénase en général, avec transfert d'un groupement hydroxyle) d'un grand nombre de composés, endogènes ou xénobiotiques (médicaments) (**Figure 9**). Ce groupe d'enzymes est caractérisé par une forte homologie de séquence au sein d'une même espèce et entre différentes espèces (notion de phylogénie)[72].

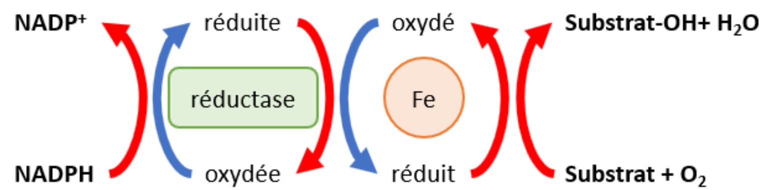

Figure 9 : Principe général de la réaction de monoxygénation catalysée par les CYP450
Le $NADP^+$ est réduit en NADPH, apportant les électrons nécessaires à la réduction de réductase(s) qui va (vont) secondairement transférer ces électrons sur l'atome de fer (Fe) contenu dans la molécule d'hème du CYP450 (le fer ferrique Fe^{3+} devient fer ferreux Fe^{2+}). L'atome de fer ainsi réduit (fer ferreux) va pouvoir activer une molécule de dioxygène afin de transférer un groupement hydroxyle (-OH) sur le substrat.

Elles ont en commun l'organisation générale de leur structure : une apoprotéine reliée par un groupement cystéinate (résidu Cystéine) à une molécule d'hème centrée par un atome de fer (**Figure 10**). La partie protéique est formée d'une séquence d'environ 500 acides aminés (poids de 50 à 60 kDa). Douze hélices α (nommée de A à L) et quatre feuillets β reliés par des boucles délimitent une cavité catalytique hydrophobe. L'extrémité carboxy-terminale comprend le résidu cystéine pour la liaison à l'hème, localisé dans une région particulière nommée *Cys-pocket*, et des sites d'interaction aux cofacteurs. L'extrémité amino-terminale permet l'ancrage de la protéine dans la bicouche lipidique de la membrane microsomale ou mitochondriale et forme le canal emprunté par le substrat pour rejoindre le site catalytique.

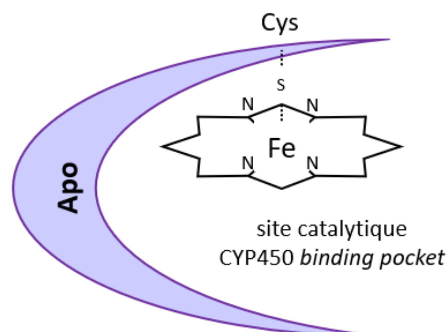

Figure 10 : Représentation schématique de l'organisation de la structure d'un CYP450
Apo : Apoprotéine. Cys : cystéine.

Il existe deux sous-types principaux de CYP450 caractérisés par leur localisation cellulaire : les CYP450 microsomaux localisés dans des vésicules issues du réticulum endoplasmique et les CYP450 mitochondriaux localisés à la face profonde de la membrane mitochondriale interne. Les premières fonctionnent grâce à la NADPH-cytochrome réductase tandis que les secondes utilisent des cofacteurs supplémentaires, la ferrédoxine et la ferrédoxine réductase.
La position de l'atome de carbone sur lequel sera transférée la fonction hydroxyle dépend de sa distance par rapport à l'atome de fer inclus dans la molécule d'hème. On définit ainsi le concept de régiosélectivité : c'est la conformation tridimensionnelle de la cavité enzymatique qui définit la position du carbone cible, et donc la fonction de l'enzyme. Lorsqu'un seul type de substrat ne peut occuper qu'une seule « position » dans la cavité enzymatique, il n'y a qu'une seule réaction d'oxydation possible sur un atome de carbone précis. Au contraire, si plusieurs substrats peuvent être métabolisés, ou si un substrat peut occuper plusieurs « positions » dans la cavité enzymatique, alors différentes réactions sont possibles. De même, une variation dans la séquence d'acides aminés composant la partie protéique peut modifier sa conformation tridimensionnelle et donc sa régiosélectivité, aboutissant à une perte ou un gain de fonction.

Activation : hydroxylation de la vitamine D en position 25

La première étape de l'activation de la vitamine D a lieu dans le foie. Il s'agit de l'ajout d'une fonction hydroxyle au niveau de l'extrémité de la chaîne latérale, sur le carbone en position 25, pour former la 25-hydroxyvitamine D (25-OH-D) ou calcidiol, la forme de vitamine D la plus abondante dans le plasma (concentration mille fois plus élevée que celle de la 1,25-$(OH)_2D$). Ce métabolite sert de forme de stockage et de transport. Il est métaboliquement peu actif. Sa demi-vie est de l'ordre de 3 à 4 semaines.
En pratique médicale courante, l'analyse du taux sérique de 25-OH-D est le critère biologique d'évaluation du stock en vitamine D. Selon les recommandations du GRIO (Groupe de Recherche et d'Information sur les Ostéoporoses) actualisées en août 2011[73], ce taux se situe idéalement chez l'adulte entre 30 et 70 ng/mL (ou 75 et 175 nmol/L, 1 nmol/L= 0,4 ng/mL). En dessous de 30 ng/mL (ou 75 nmol/L), on parle d'insuffisance vitaminique, et en dessous de 10 ng/mL (ou 25 nmol/L), de carence vitaminique.
Différentes enzymes capables de catalyser la réaction de 25-hydroxylation de la vitamine D ont pu être identifiées, que ce soit chez l'Homme (CYP2R1, CYP27A1, CYP2J2, CYP3A4, …) ou dans le règne animal (CYP2C11 chez le rat mâle, CYP2D25 chez le cochon), certaines de ces enzymes n'ayant par ailleurs pas leur équivalent chez l'Homme. Ainsi, une question restée

longtemps en suspend a été celle de l'identification de l'enzyme principalement responsable de la 25-hydroxylation de la vitamine D en condition physiologique.

En 2004, l'étude de patients présentant une forme rare de rachitisme vitamino-résistant à 25-OH-D indétectable (rachitisme pseudocarentiel de type 1B (VDDR1B)) décrite 10 ans plus tôt a permis l'identification d'une variation perte de fonction de *CYP2R1*, indiquant que ce gène codait vraisemblablement l'enzyme de la 25-hydroxylation[74–76]. Cette hypothèse est renforcée par la publication de quelques familles supplémentaires[77–79] et par l'association de polymorphismes localisés dans la région 5' non transcrite en amont de l'exon 1 de *CYP2R1* (rs2060793, rs10741657) avec des taux sériques de 25-OH-D plus faibles dans plusieurs études de type GWAS (*Genome Wide Association Study*)[80].

L'enzyme vitamine D 25-hydroxylase codée par le gène *CYP2R1* (11p15.2) a été identifiée en 2003[81]. Il s'agit d'une protéine homodimérique microsomale de 501 acides aminés capable de métaboliser les vitamines D_2 et D_3[81], sous forme non hydroxylée ou 1α-hydroxylée, mais pas les formes 25-hydroxylées, ni le cholestérol ou le 7-DHC[81] (**Figure 11**). Elle est fortement exprimée dans le foie, mais aussi dans les testicules, probablement en raison de son rôle dans la régulation de la prolifération cellulaire[81]. Sa séquence est fortement conservée à travers les espèces[72,82], en rapport avec sa forte régiosélectivité : elle ne réalise que la 25-hydroxylation. L'ajout de fonction hydroxyle sur d'autres carbones de la chaîne latérale de la vitamine D n'est pas possible en condition physiologique. C'est une enzyme de faible capacité mais de forte affinité, elle est donc active en présence de faible concentration de substrat, de l'ordre du nanomolaire.

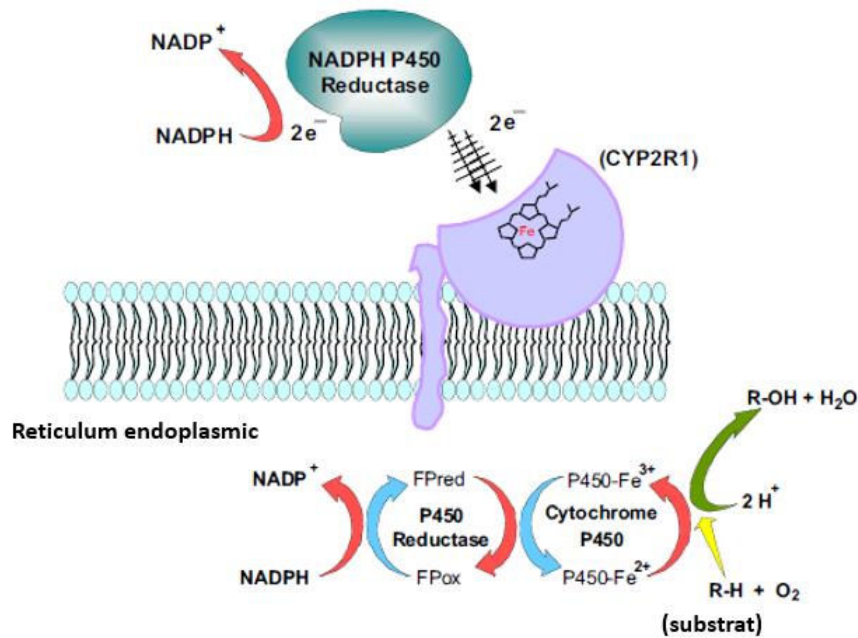

Figure 11 : Oxydation de la vitamine D par la 25-hydroxylase microsomale CYP2R1
FPred : réductase réduite ; FPox : réductase oxydée ; R : substrat

L'étude de modèles murins Cyp2r1$^{-/-}$ montre un taux de 25-OH-D plus faible d'environ 40 % mais ces animaux ne présentent ni hypocalcémie, ni rachitisme, ce qui souligne les différences entre métabolisme humain et murin et suggère l'existence d'autre(s) 25-hydroxylase(s) plus active(s) chez la souris[83]. Ce modèle n'est donc pas complètement adapté à la description des mécanismes physiopathologiques impliqués chez l'Homme.

Activation : hydroxylation de la vitamine D en position 1α

L'ajout d'une fonction hydroxyle au niveau du cycle A, sur le carbone en position 1α, crée la 1,25-$(OH)_2$D. Cette forme « hormonale » de la vitamine D possède la plus grande affinité pour le récepteur et est donc considérée comme métaboliquement active (500 fois plus active que la 25-OH-D). Sa demi-vie est faible, de quelques heures, de même que sa concentration, de l'ordre du picomolaire (pmol/L).

En pratique médicale, le dosage de la 1,25-$(OH)_2$D n'est habituellement réalisé qu'en cas de maladie du métabolisme phosphocalcique. Les valeurs normales de ce type de dosage varient en fonction de la saisonnalité, de l'âge du sujet, du type de dosage employé.

Cette réaction n'est catalysée que par une seule enzyme, la 25-hydroxyvitamine D 1α -hydroxylase codée par le gène *CYP27B1* (12q14.1)[84–86] à partir de la 25-OH-D_2 et de la 25-OH-D_3. Cette enzyme mitochondriale de 507 acides aminés[87] possède une expression quasi

ubiquitaire en rapport avec une synthèse de 1,25-$(OH)_2D$ dans de nombreux organes et tissus cibles de la vitamine D (reins, ostéoblastes, kératinocytes, colon, système hématopoïétique et placenta (syncytiotrophoblaste))[88–97] (**Figure 12**).

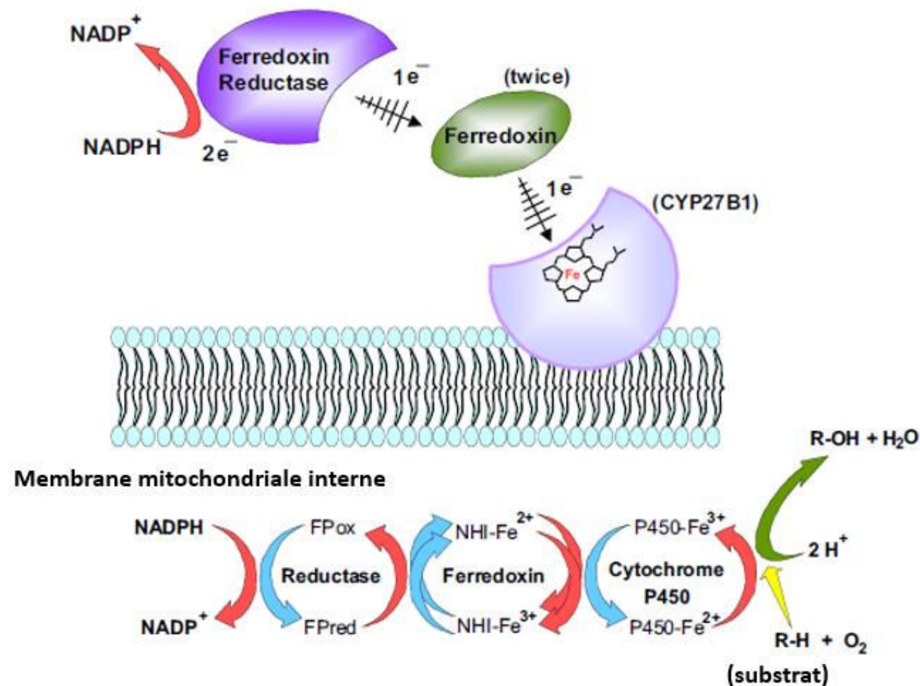

Figure 12 : Oxydation de la vitamine D par la 1α-hydroxylase mitochondriale CYP27B1
FPred : réductase réduite ; FPox : réductase oxydée ; R : substrat

En condition physiologique, l'homéostasie phosphocalcique dépend exclusivement de la régulation de la 1,25-$(OH)_2D$ sérique produite grâce à la forte expression rénale de *CYP27B1*[98–100]. Cela explique une diminution de cette concentration chez les patients insuffisants rénaux et dans les modèles animaux néphrectomisés. Au contraire, la production extrarénale de 1,25-$(OH)_2D$ a vraisemblablement un rôle dans la régulation du métabolisme cellulaire (prolifération en particulier), ce qui expliquerait les nombreuses associations statistiques décrites entre concentration sérique en 25-OH-D et maladies (cardiovasculaires, auto-immunes, cancers, …)[51,101,102]. Dans les syndromes lymphoprolifératifs (lymphomes, sarcoïdoses, granulomatoses), une expression extrarénale massive de *CYP27B1* peut aboutir à une hypercalcémie par production excessive de 1,25-$(OH)_2D$.

Le phénotype de rachitisme vitaminorésistant à 1,25-$(OH)_2D$ basse (rachitisme pseudocarentiel de type 1A) présenté par les patients porteurs de mutations bialléliques perte de fonction de *CYP27B1* décrit en 1997 [84] suggère l'absence d'enzyme accessoire capable de catalyser la 1α-

hydroxylation de la vitamine D. L'observation de modèles murins Cyp27b1$^{-/-}$ avec phénotype similaire est cohérente avec cette hypothèse[103,104]. En outre, ces animaux présentent une diminution du nombre de lymphocytes CD4+ et CD8+ et une infertilité chez les souris femelles[105].

Dégradation : hydroxylation de la vitamine D en position 24

L'enzyme mitochondriale responsable de la dégradation de la vitamine D est la vitamine D 24-hydroxylase codée par *CYP24A1* (20q13.2), gène dont la séquence est particulièrement conservée entre les espèces[106]. Cette enzyme catalyse plusieurs étapes d'hydroxylations séquentielles qui permettent l'oxydation de la 25-OH-D et de la 1,25-$(OH)_2D$[107]. A l'image de *CYP27B1*, *CYP24A1* est exprimé dans les reins mais également dans de nombreux tissus (os, cœur, poumons, intestins, pancréas, rate, …) où son expression augmente en cas de traitement par 1,25-$(OH)_2D$[107,108] pour réguler la quantité de 1,25-$(OH)_2D$ à l'échelle de l'organisme (métabolisme phosphocalcique) ou de la cellule (régulation de la prolifération/différentiation) respectivement[109].

Il existe deux voies de dégradation de la vitamine D définie par la position de l'atome de carbone de la chaîne latérale supportant la première réaction d'hydroxylation, soit C23 (la première réaction d'hydroxylation a lieu en position 23), soit C24 (la première réaction d'hydroxylation a lieu en position 24)[107] (**Figure 13**). La propension d'un organisme à utiliser l'une ou l'autre de ces voies (définissant le rapport C24/C23) dépend de la séquence d'acides aminés qui constitue l'apoprotéine (concept de régiosélectivité vu précédemment) et est donc variable d'une espèce à l'autre. Ainsi, le résidu Glycine en position 326 chez le rat oriente principalement le catabolisme de la vitamine D vers la voie C23, tandis que la substitution de cet acide aminé par une Alanine, comme dans la protéine humaine, l'oriente vers la voie C24[110]. Les fonctions respectives de ces différentes voies métaboliques, si elles existent, ne sont pas actuellement connues[110].

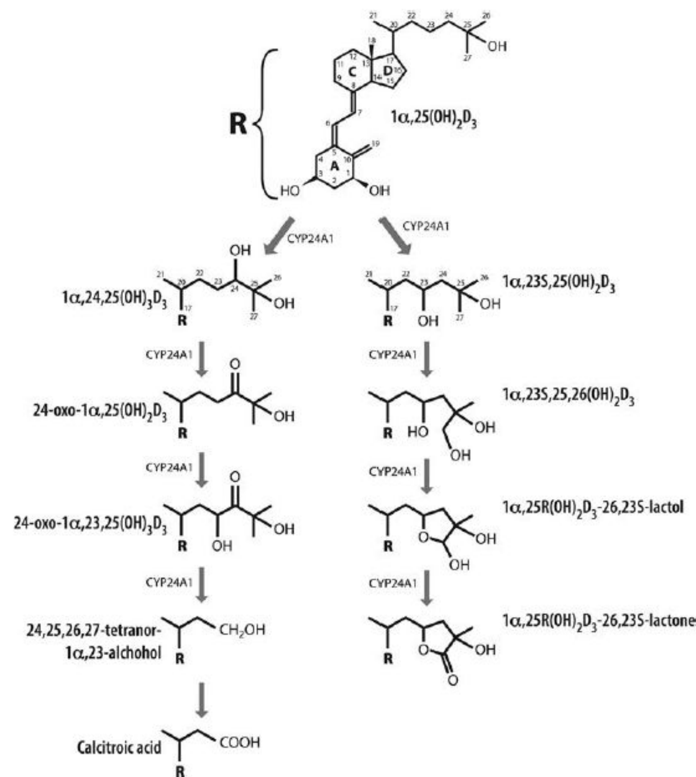

Figure 13 : Représentation schématique des deux voies de dégradation de la vitamine D (1,25-$(OH)_2D$) par la 24-hydroxylase, C23 (à droite) et C24 (à gauche) (d'après *Vitamin D 4th Edition*, chapitre *CYP24A1: Structure, Function, and Physiological Role*, R. St-Arnaud et G. Jones)

La voie C24 est la voie de dégradation principale chez l'Homme (rapport C24/C23 d'environ 3,7). Elle comporte cinq réactions d'hydroxylation dont la première permet une baisse d'affinité de la 1,25-$(OH)_2D$ (devenue 1,24,25-$(OH)_3D$) pour le VDR d'un facteur 10. Elle s'achève avec la libération de la chaîne latérale et l'oxydation du produit de clivage pour former l'acide calcitroïque. La voie C23 aboutit après quatre étapes à la production de 1α,25R$(OH)_2D$-26,23S-lactone[111,112]. Les produits de dégradation sont excrétés dans la bile[113,114].

La vitamine D 24-hydroxylase réalisant l'oxydation de chaque produit sur l'une ou l'autre de ces voies (oxydation séquentielle), chaque métabolite intermédiaire est libéré dans le torrent sanguin. Parmi ces molécules, le métabolite sanguin dihydroxylé le plus abondant est la 24,25-$(OH)_2D$ (concentration de l'ordre du nanomolaire) dont les effets biologiques ne sont pas actuellement connus, bien qu'un rôle dans la consolidation des fractures osseuses ait été suggéré[115].

En 2011, suivant une stratégie dite de gène candidat, des variations pathogènes perte de fonction de *CYP24A1* ont été associées à une forme autosomique récessive d'hypercalcémie infantile idiopathique ou hypercalcémie par mécanisme d'hypersensibilité à la vitamine D[115].

Régulation du métabolisme de la vitamine D

Comme vu précédemment, la régulation du métabolisme de la vitamine D vise à maintenir les quantités nécessaires en 1,25-$(OH)_2$D active. La production de 25-OH-D n'étant pas régulée, cette régulation s'articule autour de deux grands axes : la seconde étape d'activation (1α-hydroxylation) et la dégradation (24-hydroxylation) (**Figure 14**). Les facteurs régulant ces mécanismes diffèrent en fonction de l'échelle considérée, échelle de l'individu (régulation du métabolisme phosphocalcique) ou de la cellule (régulation de la prolifération).

A l'échelle de l'organisme entier, le métabolisme de la vitamine D dépend principalement du métabolisme du calcium et du phosphate[116,117]. Les expressions rénales de *CYP27B1* et *CYP24A1* sont régulées de façon coordonnées et inversées par différentes hormones impliquées dans le métabolisme phosphocalcique. La PTH stimule l'expression de *CYP27B1* (action sur le tubule contourné proximal) et diminue la production de 24-hydroxylase en favorisant la dégradation de l'ARNm[118]. La 1,25-$(OH)_2$D inhibe l'expression de *CYP27B1* et stimule celle de *CYP24A1* par un mécanisme dépendant du VDR. Le FGF23 (*Fibroblast Growth Factor 23*), principale hormone régulatrice du métabolisme du phosphate[119–124], régule l'expression de transporteurs tubulaires de phosphate mais également celle de *CYP27B1* et *CYP24A1*, qu'il inhibe et stimule respectivement[125–127]. Ainsi, il existe plusieurs boucles de rétrocontrôle (calcémie et PTH, 1,25-$(OH)_2$D et VDR, phosphatémie et FGF23) qui permettent une régulation fine de la concentration sanguine de 1,25-$(OH)_2$D.

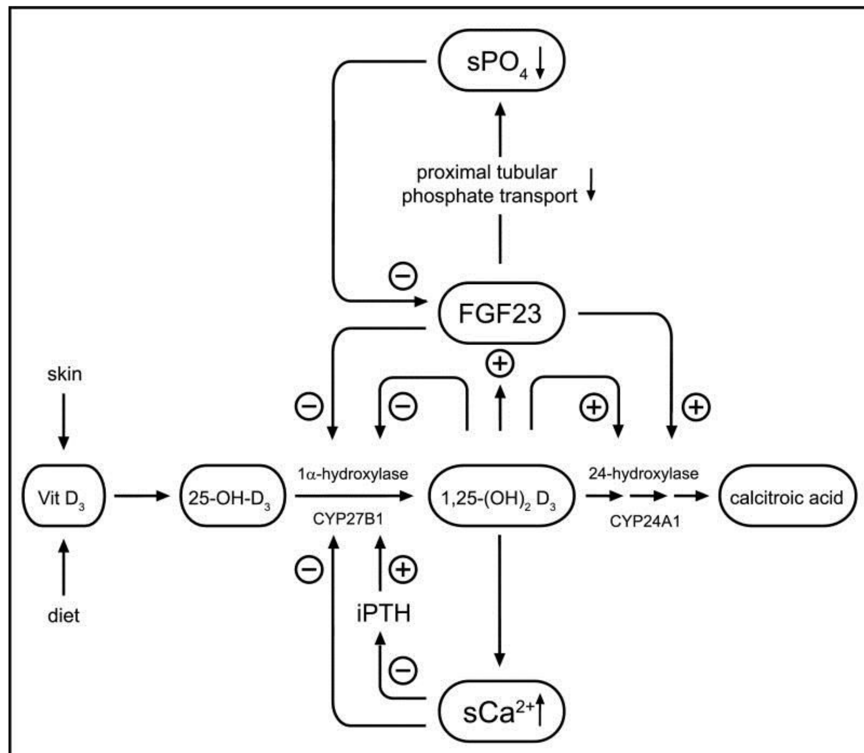

Figure 14 : Vitamine D et métabolisme phosphocalcique
(d'après Schligmann et al. *J Am Soc Nephrol* 2016; 27(2): 604–614)

Les modalités de régulation du métabolisme de la vitamine D dans le tissu extrarénal sont moins connues, vraisemblablement sous l'influence de facteurs tissus spécifiques (actions des cytokines sur les leucocytes comme l'interféron-γ qui favorise l'expression de *CYP27B1* dans les macrophages, variant inactif spécifique de la 24-hydroxylase propre aux monocytes et macrophages[128]).

Mécanismes moléculaires impliqués dans la régulation du métabolisme de la vitamine D par la 1,25-$(OH)_2D$

Les mécanismes moléculaires à l'origine de la régulation inverse et coordonnée de l'expression de *CYP27B1* et *CYP24A1* dans le rein sont mal connus. À la suite de l'identification de ces gènes et de leur localisation, des efforts ont été déployés afin d'étudier les séquences localisées immédiatement en amont du site d'initiation de la transcription (TSS) afin de caractériser leur promoteur proximal. Les approches employées combinaient l'utilisation d'outils bioinformatiques d'analyse de séquences, afin de localiser des éléments de liaison aux facteurs de transcription putatifs, et des études fonctionnelles *in vitro*, avec clonage, fragmentation et

inclusion de séquences supposées régulatrices dans des modèles cellulaires afin d'en caractériser les différentes parties et effets [129,130].

Ainsi, le promoteur proximal de *CYP27B1* (**Figure 15A**) contient une boite TATA, une boite CAAT et différents sites de liaison, entre autres, aux facteurs de transcription de type CREB (*C-AMP Response Element-binding protein*, facteur de transcription liant l'AMP cyclique, second messager dont la production intracellulaire augmente suite à l'activation de l'adénylate cyclase par la protéine Gsα couplée au récepteur de la PTH), mais également NF-κB (voie de signalisation impliquée dans la réponse immunitaire et inflammatoire et la régulation de l'apoptose). Il n'a pas été localisé de VDRE classique en amont de ce gène et la séquence des nVDRE n'étant pas caractérisée, les sites de liaison du VDR au niveau du promoteur proximal de *CYP27B1* ne sont pas identifiés.

Les études de caractérisation du promoteur proximal réalisées par Murayama[129] et Kong[130] dans des modèles cellulaires différents (cellules dérivées de tubule contourné proximal de souris MCT et cellules épithéliales rénales de porc AOK-B50 respectivement) ont abouti à des conclusions différentes. La première étude concluait à l'existence d'éléments de réponse à la PTH (activateur) et à la 1,25-$(OH)_2D$ (répresseur) respectivement localisés environ 4 kb et 0,5 kb en amont du TSS, identifiés grâce à leur inclusion dans une construction de type pGL-tk-CAT (plasmide contenant les fragments étudiés, le promoteur proximal de la thymidine kinase et le gène codant la chloramphénicol acétyltransférase comme gène rapporteur). La seconde étude, basée sur l'utilisation d'une construction plus simple (fragments de promoteurs inclus dans un plasmide pGL2 en amont du gène codant la luciférase, conservant la partie la plus proximale du promoteur), concluait à l'existence d'éléments répresseurs principalement localisés sur le fragment AN4 et d'éléments activateurs sur les fragments AN2 et AN5 principalement (**Figure 15B**).

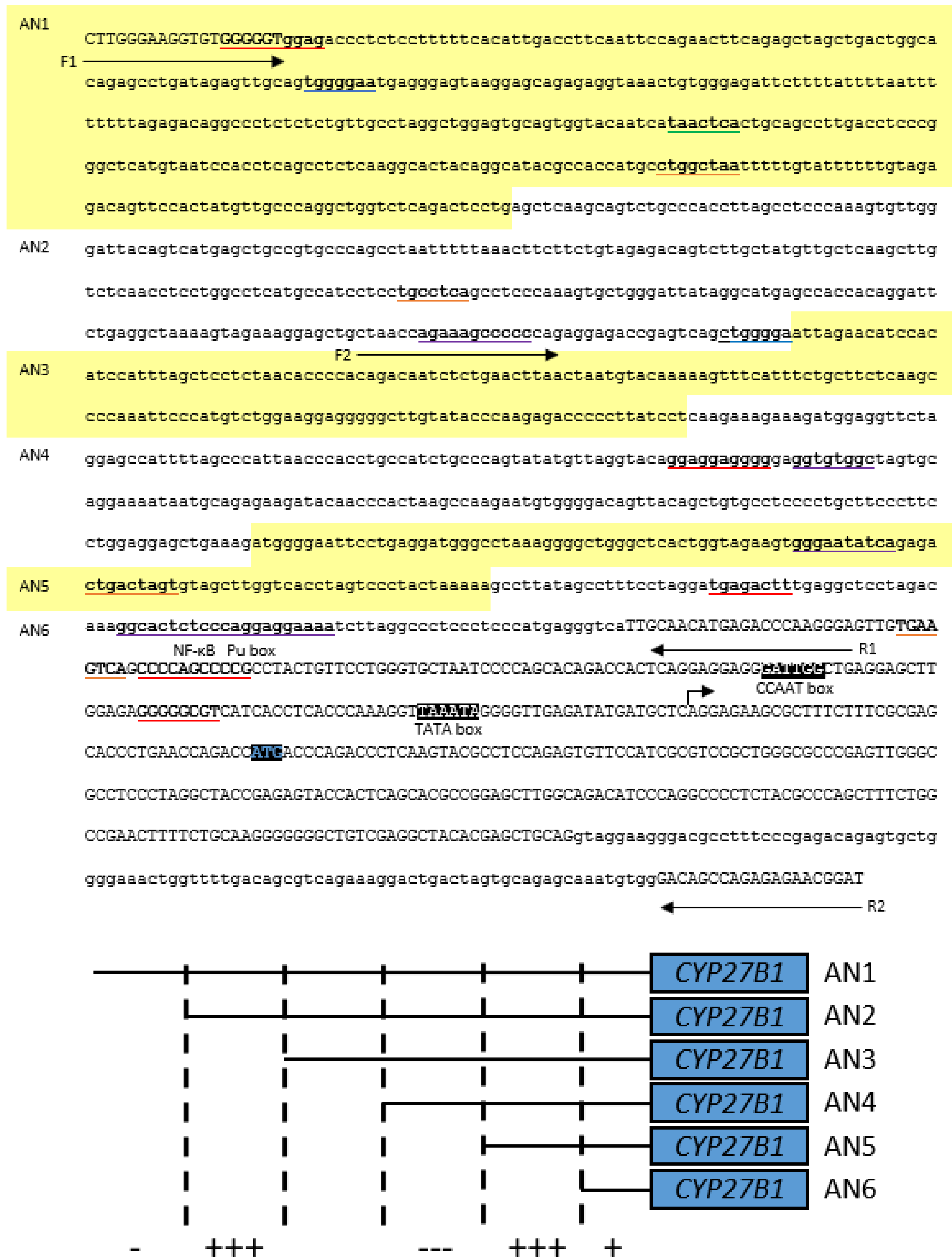

Figure 15 : Séquence du promoteur proximal de *CYP27B1* et fragments étudiés par Kong *et al.*

Sites de fixation aux facteurs de transcription déterminés par analyse bioinformatique (rouge : Sp1, bleu : AP-2, vert : AP-1, orange : cAMP Responsive Element, violet : NF-κB)

De la même façon, au niveau du promoteur proximal de *CYP24A1*, la présence de VDRE localisés directement en amont du TSS a été mise en évidence assez tôt par différentes équipes chez le rat [33–36] puis chez l'Homme[131]. Ces éléments sont responsables de l'activation de la transcription de *CYP24A1* par la 1,25-$(OH)_2D$ après fixation de l'hétérodimère RVR-RXR. Il existe néanmoins des différences significatives entre les promoteurs proximaux de ces deux espèces, notamment l'existence d'un élément spécifique présent chez le rat nommé VSE pour *Vitamin D stimulating element* (séquence 5'-TGTCGGTCA-3' liant un facteur de transcription indéterminé) et non fonctionnel chez l'Homme (**Figure 16B**)[132].

En 2008, les travaux de Roff et Wilson portèrent sur un SNP (rs111622401) localisé dans la séquence du VDRE2 présent en amont du TSS de *CYP24A1* identifié à l'état hétérozygote chez des patients Afro-Américains[133] (**Figure 16A**). Ces auteurs associèrent ce SNP à une diminution de l'expression de *CYP24A1* induite par la 1,25-$(OH)_2D$ dans des cellules H1299 (lignée de cancer pulmonaire) et MCF-7 (lignée de cancer du sein). Ainsi, leur observation valide l'hypothèse selon laquelle une modification de la séquence d'un VDRE pourrait être à l'origine d'une perturbation de la régulation de l'expression de *CYP24A1* chez l'Homme. Ils s'interrogèrent d'ailleurs sur les conséquences d'un tel variant à l'état homozygote, ne l'ayant identifié qu'à l'état hétérozygote dans une petite cohorte de patients.

-290 to -276
VDRE 2
-170 to -147
VDRE 1
CYP24A1
▽ = Previously known
▼ = Novel
GAGTTCaccGGGTGT
GAGTCAgcgAGGTGAgcgAGGGCG

```
CTGGCACCTCTCGCGGCTGAGGGGACGTGTACGCCGTAGATGTCACCAGTCTCGGGGGCTGCCTCGGACTGCGCAG
CTGCTGCAGGAAGGCGGCAAGCGAGCGGCTCTTGCTGATGGGGGAGCTCATGGCAGCGGGGGACACCGGAGCGCGG
GAAGGCAGGAGGATGGGGTGGGGCGAGGTTGGTACGAGGTGCTAGTGGGAGTCGGGGCTTAACGATTCTGGGAAAA
GGAAGCAAAGAGGGCCAGCTGGTGTGATGGAGCGGGGTGAGGCGGGACAGGCAGCAGAAAGGACCTCGGCGAGGAT
GCTCGACGCTGCACCACGCGACAGCCTCAGAGCATTGGTGCCTCCTTGCACTGGCCGCAGGGGCTGGAAGAGGGTG
GCCGGTGTCTGGGGACCTCTTGAAAAGGGAAAGCTGGGAGTCCTCCTGTTGGTCCGCAAGGCTGACCTCTAGGGTC
TGGCTGGAGCCACGGGGAGGTGTCAAGGAGGGTAGATGAGATGCTGCTGGCGCTGCGTTTCCTCCTGTCcctctcc
                 F
atgttcctatgcccagggaccatgcatttatggagacagagcatgccctgggtggccaatgagcacgcagaggagg
gcggagtggcccccggctggccagactccgagggacgcgggacccaggggagggggggtggaaggaggatgGAGTC
AgcgAGGTGAgcgAGGGCGtccgggcctgggtgaccggggctatgttcgctgggcgcgggaggtggggtgggggc
    VDRE 1                   VSE
ggcagactccaccccggagataacccccaggaagtcatgcgaagcccgGAGTTCaccGGGTGTgcttcgaacgcgc
                                                   VDRE 2
ctccccgccctcccccggcgggcaggagccggcagcggcggccgtggttgccgcccggcgcttgcacaatcgcgat
                                                                 C/EBP
gccgggcgggcgaggtccccgagtgcgggcccagaaaagctgccggcgtctgccagggccccggggagcccgggcg
cctcccggggagccagataccccc agcctggactgaagtgatttttttttttttttgcctttttttttttggagg
aagtttggctttctgcgcctcccagggtggggaggatttgagtcttactgaaatgcaccagaaGAGGAGACTTTCT
TAACTTGAGCAA R
```

Figure 16 : Représentation schématique et séquence du promoteur proximal de *CYP24A1*
Les triangles jaunes et rouges représentent des SNP identifiés dans la séquence du promoteur.
(d'après Roff et Wilson *J Steroid Biochem Mol Biol* 2008)

Plus récemment, l'utilisation combinée de technique d'immunoprécipitation de la chromatine couplée à l'hybridation génomique comparative sur puce à ADN (ChIP-Chip) ou au séquençage (ChIP-seq) ont permis la mise en évidence de régions régulatrices localisées à plusieurs kb des promoteurs de *CYP27B1* et *CYP24A1*[39,134].

En 2010, Meyer et collaborateurs[39] appliquèrent ces techniques sur des cellules humaines (lignée colique LS180) et murines (lignée ostéoblastique MC3T3-E1) avec des anticorps anti-VDR et anti-RXR. Ils identifièrent des régions régulatrices localisées plusieurs kb en aval de *CYP24A1*, contenant des VDRE. Des études fonctionnelles couplant mutagénèse dirigée et inclusion de ces séquences régulatrices dans des constructions de type BAC suggèrent que des

altérations (mutation ou délétion) de ces régions peuvent être responsables d'une diminution, voire d'une suppression de l'expression de *CYP24A1* induite par la 1,25-$(OH)_2D$. Enfin, des études complémentaires de type 3C (*Chromosome Conformation Capture*) et par ChIP-Chip à l'aide d'anticorps spécifiques (anti-ARN polymérase de type 2, anti-histone H4 acétylée, anti-facteur CTCF) permirent de préciser l'architecture chromatinienne autour de *CYP24A1*, suggérant l'existence d'un TAD (*Topologically Associated Domain*) d'environ 110 kb (**Figure 17**) et de boucles chromatiniennes permettant *in vivo* une proximité physique entre le promoteur proximal et les régions régulatrices distales.

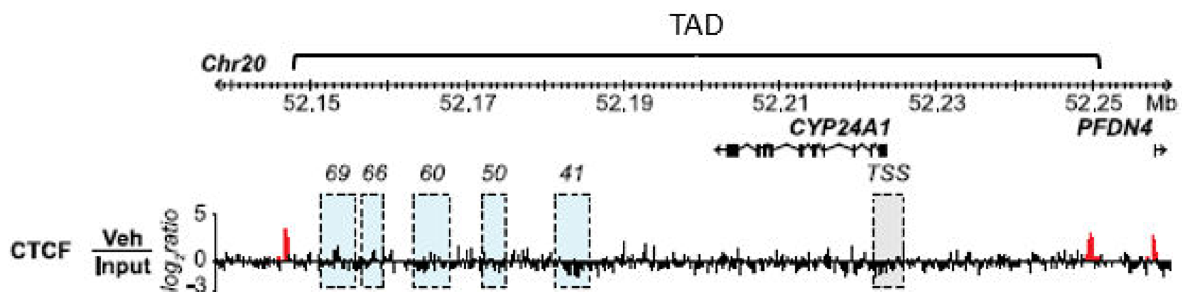

Figure 17 : Représentation schématique du TAD de *CYP24A1* suggéré par Meyer *et al.*
En rouge, frontières de TAD supposées par l'analyse ChIP-chip avec anticorps anti-CTCF. En bleu, éléments régulateurs distaux. En gris, promoteur proximal.

Le même type de procédures furent utilisées par ces mêmes auteurs pour caractériser chez la souris une région de 16 Mb, correspondant vraisemblablement à un TAD incluant les gènes Cyp27b1, Mettl1 et Mettl21b (**Figure 18**). Plusieurs régions régulatrices furent également associées au contrôle de l'expression de Cyp27b1 par le FGF23 (élément régulateur localisé dans un intron de Mettl21b), la PTH (élément régulateur localisé dans un intron de Mettl1) et la 1,25-$(OH)_2D$ (les deux sites sus-menstionnées seraient potentiellement impliqués) dans le rein. Une approche par *genom editing* utilisant la technologie CRISPR/Cas9 leur permit de générer des souris présentant des délétions de ces zones régulatrices afin de confirmer leur rôle dans la régulation de l'expression de la 1α-hydroxylase rénale. L'étude de l'expression des gènes du métabolisme de la vitamine D dans des cellules extrarénales de ces mêmes animaux suggèrent que les régions promotrices précédemment identifiées n'ont aucun rôle en dehors du rein.

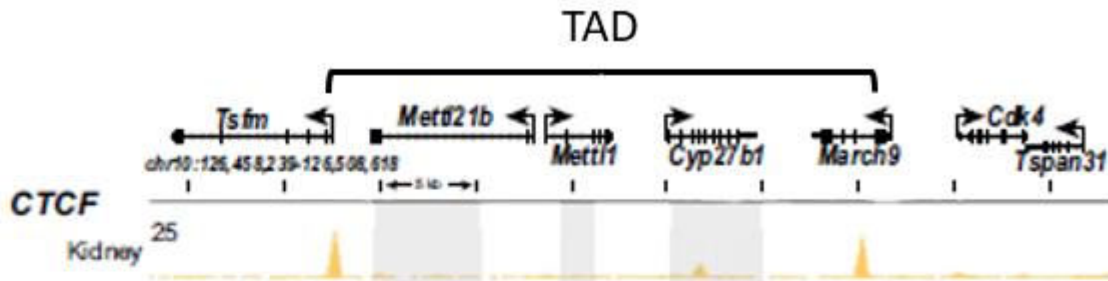

Figure 18 : Représentation schématique du TAD de *CYP27B1* suggéré par Meyer *et al.* En rouge, frontières de TAD supposées par l'analyse ChIP-chip avec anticorps anti-CTCF. En bleu, éléments régulateurs distaux. En gris, promoteur proximal.

Ainsi, sur le plan moléculaire, la régulation coordonnée de *CYP27B1* et *CYP24A1* repose sur l'organisation tridimensionnelle de la chromatine à ces loci et sur l'activité de régions régulatrices tissu-spécifiques, en partie identifiées chez la souris mais non déterminées chez l'Homme. Des altérations génomiques n'affectant pas la séquence codante de ces gènes mais modifiant ces régions (perturbation de l'organisation des TAD, modification de la séquence des régions régulatrices) représentent donc des causes potentielles de dérégulation du métabolisme de la vitamine D d'origine génétique.

Pathologie associée aux anomalies du métabolisme de la vitamine D

Carence en vitamine D et rachitisme

Le rachitisme est une maladie de l'enfant liée à un défaut de minéralisation du squelette en croissance (plaques de croissance) secondaire à une carence en calcium et/ou en phosphate. Le phosphate étant abondant dans l'alimentation, la carence en phosphate n'est que rarement à l'origine de rachitisme (en dehors du cadre de maladies génétiques spécifiques). La carence nutritionnelle en calcium (apports inférieurs à 300 mg/j)[135,136] est également rare, principalement observée dans les pays en voie de développement [137–143] (régime alimentaire infantile caractérisé par des céréales pauvres en calcium et un faible contenu en produits laitiers)[135,144–146].

En revanche, le rachitisme par carence en vitamine D fut quasi endémique en Europe de la fin du XIXème siècle au début du XXème, jusqu'à la généralisation de la supplémentation. De nos jours, il persiste dans les pays en voie de développement (faible accès à la supplémentation ou

aux produits enrichis), sous l'influence de facteurs socio-culturels (*purdah*, alimentation vegan stricte excluant les produits laitiers enrichis, allaitement prolongé) ou chez les populations migrantes (populations à phototype foncé migrant vers des zones à faible rayonnement UVB)[147–157].

La maladie se manifeste lorsque la concentration sérique en 25-OH-D chute sous les 30 à 125 nmol/L [158–160], selon les apports calciques et l'exposition aux rayonnements UVB. Trois stades d'évolution de la maladie ont été décrits par Fraser *et al.*[161] :

- le stade 1 correspond à des manifestations inconstantes et aspécifiques de l'hypocalcémie dues à une diminution de l'absorption intestinale de calcium, qui stimule la sécrétion de PTH[162] visant à élever la concentration en 1,25-$(OH)_2$D pour augmenter l'absorption intestinale et la réabsorption tubulaire de calcium ainsi que la résorption osseuse (d'où une élévation des phosphatases alcalines) afin de maintenir une calcémie normale,
- le stade 2 correspond à la disparition de ces signes d'hypocalcémie avec apparition des déformations osseuses caractéristiques : l'hyperparathyroïdie chronique conduit à une hypophosphatémie secondaire avec hyperphosphaturie responsable des troubles de la minéralisation des plaques de croissance,
- le stade 3 correspond à la réapparition des signes d'hypocalcémie chez un enfant présentant une atteinte squelettique une fois les mécanismes de compensation dépassés.

Il existe bien entendu un chevauchement entre ces trois stades.

Sur le plan clinique, la maladie se manifeste le plus souvent à un âge compris entre 3 et 18 mois[163] mais des formes néonatales ou congénitales ont été décrites chez des enfants nés de mères carencées [164–169] (avant 3 mois, la concentration sérique en 25-OH-D du nouveau-né dépend de la concentration maternelle car cette vitamine traverse la barrière placentaire[170]). L'allaitement par une mère carencée en vitamine D est également un facteur de risque de rachitisme carentiel précoce[170,171]. Outre les signes aspécifiques de l'hypocalcémie (apnées et convulsions, tétanie, hypotonie, décalage des acquisitions), le tableau classique de rachitisme comporte des déformations osseuses liées à la mise en charge (poids du corps) sur un squelette déminéralisé, plus visibles aux poignets et avant-bras ou aux membres inférieurs selon l'âge du patient (station à quatre pattes ou debout), volontiers associées à des douleurs (en particulier à la marche). Des lésions costales peuvent être observées sous la forme dite du chapelet rachitique (*rachitic rosary*) et d'une déformation thoracique (*violin case deformity* avec thorax étroit et

abdomen proéminant). D'autres signes osseux sont également possibles : fractures costales[172], retard à la fermeture des fontanelles, *craniotabes* (défaut de minéralisation de la voute crânienne)[173], craniosténose[174], retard à l'éruption dentaire et hypoplasie de l'émail (denture permanente)[175]. Sur le plan extraosseux, la carence en vitamine D peut s'associer à des signes musculaires (tableau pseudo-myopathique[176], cardiomyopathie[177,178]) témoins de son implication dans la physiologie musculaire, d'une susceptibilité accrue aux infections[179,180] et d'anomalies hématologiques[181] témoins de ses effets dans la régulation du système hématopoïétique et immunitaire.

Sur le plan biologique, on retiendra que le tableau précoce peut être faussement évocateur d'hypoparathyroïdie ou de pseudohypoparathyroïdie (hypocalcémie, hyperphosphaturie, phosphatases alcalines normales avant la réaction parathyroïdienne). Il se modifie avec l'évolution de la maladie, facilitant le diagnostic (hyperparathyroïdie, élévation des phosphatases alcalines) et sur le plan urinaire (hypocalciurie, augmentation de l'excrétion d'AMPc et de l'excrétion d'acides aminés, défaut d'excrétion d'acides). Concernant les métabolites de la vitamine D, outre la concentration basse de 25-OH-D à la base du diagnostic, on observe une concentration faible voire indétectable de 24,25-$(OH)_2D$ [182–184] et une concentration de 1,25-$(OH)_2D$ variable selon le stade de la maladie (basse, normale ou haute) [182–186], la plus élevée lors du stade 2, mais toujours inadaptée (insuffisamment élevée en regard de la calcémie).

Les signes radiologiques du rachitisme sont bien visibles dès le stade 2 de la maladie, en particulier à l'extrémité distale de l'ulna chez le jeune enfant[187], puis au genou chez l'enfant plus âgé. Chez les adolescents, la radiographie de bassin est utile du fait de la fusion des métaphyses des os longs (les centres d'ossification du bassin fusionnent tardivement, entre 15 et 25 ans)[188]. Il s'agit principalement d'un élargissement associé à un aspect flou et peigné des métaphyses, témoin de la désorganisation des plaques de croissance (défaut d'apoptose des chondrocytes hypertrophiques lié à l'hypophosphatémie). D'autres signes peuvent également être observés : ostéopénie avec diminution de l'épaisseur des corticales, simplification de la trame osseuse trabéculaire, incurvation diaphysaire des os longs, fractures et pseudo-fractures (lignes de Looser).

Le traitement habituellement employé est basé sur l'administration quotidienne par voie orale (en l'absence de malabsorption des graisses) de vitamine D_2 ou D_3 à la dose de 2000 à 6000

UI/jour[189] (ou 50 000 UI/semaine[190], ou dose unique de 150 000 à 600 000 UI orale ou intramusculaire[191–194], éventuellement associée à une supplémentation en calcium. Il conduit à une importante et rapide augmentation de la concentration sérique de 1,25-$(OH)_2D$ (jusqu'à 5 fois la normale après 1 à 3 semaines et jusqu'à 10 semaines après traitement [183,185]) alors que la concentration de 25-OH-D (et donc de 24,25-$(OH)_2D$) se corrige plus lentement. La calcémie, la phosphatémie et la PTH se corrige en 3 semaines [183,185]. La normalisation de la concentration de phosphatases alcalines est plus longue (6 à 12 semaines)[191].

La prévention du rachitisme repose sur des politiques de supplémentation des enfants en vitamine D et sur l'enrichissement de denrées alimentaires à l'échelle industrielle (produits laitiers et huiles végétales). Il existe de nombreuses spécialités pharmaceutiques contenant de la vitamine D. Il est important de bien distinguer la forme de la vitamine D contenue dans ces produits : non hydroxylée, mono ou dihydroxylée. Elle peut également être associée à d'autres composés, calcium ou vitamines (**Tableau 1**). La quantité de vitamine D dans chacune de ces spécialités est exprimée en Unité Internationale (UI) ou en microgramme (µg) : 100 UI correspondent à 2,5 µg, 1µg est égal à 2,5 nmol. Le choix d'un produit dépend de l'indication de la supplémentation. En France, la principale indication à la prescription de vitamine D correspond à la prévention de la carence, particulièrement dans trois populations considérées comme à risque : les nourrissons, les femmes enceintes et les sujets âgés de plus de 65 ans. Chez l'enfant de moins de 18 mois, elle est quasi systématique, démarrée tôt et enseignée aux parents dès la maternité. Chez les sujets âgés, elle peut être proposée systématiquement sans dosage préalable, les bénéfices étant multiples (prévention des chutes et complications[195,196]) et les risques faibles. La supplémentation en vitamine D est également recommandée dans d'autres affections comme l'insuffisance rénale ou l'ostéoporose.

forme isolée	association
Vitamine D_2: ergocalciférol	
UVESTEROL D, solution buvable **STEROGYL**, solution buvable en ampoule **MORISLAND**, solution buvable en gouttes	**UVESTEROL VITAMINE A.D.E.C.**, solution buvable (solution polyvitaminée) **HYDROSOL POLYVITAMINE PHARMADEVELOPPEMENT**, solution buvable en gouttes (solution polyvitaminée) **VITALIPIDE ADULTES**, émulsion injectable pour perfusion (+vitamine A) **VITALIPIDE ENFANTS**, émulsion injectable pour perfusion (+vitamine A)
Vitamine D_3 : cholécalciférol	
ADRIGYL, solution buvable en gouttes **UVEDOSE**, solution buvable en ampoule **VITAMINE D3 BON**, solution buvable en ampoule, solution injectable IM en ampoule **ZYMAD**, solution buvable	**CALTRATE VITAMINE D3**, comprimé pelliculé (+calcium) **NATECAL VITAMINE D3**, comprimé orodispersible (+calcium) **AUXERGYL D3**, solution buvable en ampoule (+vitamine A) **ZYMADUO**, solution buvable en gouttes (+fluor) **CERNEVIT**, poudre pour solution injectable ou pour perfusion (solution polyvitaminée) **SUPRADYNE**, comprimé effervescent, comprimé pélliculé (solution polyvitaminée) **ADROVANCE**, comprimé (+biphosphonate) **FOSAVANCE**, comprimé (+biphosphonate) **ACTONELCOMBI**, comprimé pelliculé et granulés effervescents (+biphosphonate +calcium)
25-hydroxyvitamine D_3 : calcifédiol	
CALCIFEDIOL RPG, solution buvable en gouttes **DEDROGYL**, solution buvable en gouttes	
1α-hydroxyvitamine D_3 : alfacalcidol	
ALFACALCIDOL TEVA, capsule molle **UN ALFA**, capsule orale, solution buvable en gouttes, solution injectable IV en ampoule	
1,25-dihydroxyvitamine D3 : calcitriol	
ROCALTROL, capsule molle	

Tableau 1 : Spécialités pharmaceutiques contenant de la vitamine D

Intoxication par la vitamine D

Si la supplémentation infantile et l'enrichissement de denrées alimentaires permirent la quasi-éradication du rachitisme dans la plupart des pays développés, des cas d'intoxication à la vitamine D furent décrits, en particulier en lien avec des campagnes de supplémentation

infantile à hautes doses ou de procédés industriels d'enrichissement du lait en vitamine D mal contrôlés (contrairement à ce qui est observé dans une majorité de pays, le lait de vache peut être enrichi aux Etats Unis et au Canada à la concentration de 400 UI/L)[197,198]. Il s'agit d'une cause peu fréquente d'hypercalcémie de l'enfant ou de l'adulte, principalement liée à un excès d'apport exogène en vitamine D, qu'il s'agisse d'une prise excessive de vitamine D native (cholécalciférol ou ergostérol) ou de 25-OH-D, ou plus rarement de 1,25-$(OH)_2$D ou d'analogues comme la 1α-OH-D ou le calcipotriol (analogue de la 1,25-$(OH)_2$D utilisé en application cutanée dans le traitement du psoriasis[199–201]). Un mécanisme d'intoxication à la vitamine D par excès de conversion de la 25-OH-D en 1,25-$(OH)_2$D est également décrit (intoxication endogène) dans des maladies associées à une surexpression de la 1α-hydroxylase en dehors du rein (granulomatoses, lymphomes, tuberculose, …). En revanche, l'excès d'exposition solaire sans protection n'expose pas au risque d'intoxication par la vitamine D chez les individus sains, l'élévation de la concentration de 25-OH-D sérique ne dépassant pas 70 à 80 ng/L (175 à 200 nmol/L)[202,203], vraisemblablement du fait de la production d'isomères biologiquement inactifs dans ces conditions.

La présentation clinique de l'intoxication à la vitamine D se résume à celle de l'hypercalcémie marquée par des signes aspécifiques (syndrome polyuropolydipsique, signes neuropsychiques : fatigue, confusion, somnolence, signes digestifs : anorexie, nausées/vomissements, constipation, signes neuropsychiatriques, troubles du rythme et de la conduction : réduction de l'intervalle QT à l'électrocardiogramme, …).

Le phénotype biologique comprend une hypercalcémie, une hypercalciurie (qui précède souvent l'hypercalcémie mais n'est pas toujours évaluée), une hyperphosphatémie[197,204,205], une PTH adaptée basse voire indétectable. Le dosage du PTHrp permet d'exclure une hypercalcémie paranéoplasique qui constitue un diagnostic différentiel principalement chez l'adulte. En cas d'intoxication par la vitamine D native ou la 25-OH-D, la concentration sérique de 25-OH-D est élevée, habituellement supérieure à 150 ng/L (375 nmol/L)[205,206], associée à une concentration sérique de 1,25-$(OH)_2$D légèrement augmentée ou normale. Au contraire, dans les intoxications (exogènes et endogènes) par la 1,25-$(OH)_2$D ou par des analogues 1α-hydroxylés, la concentration sérique de 25-OH-D est normale et celle de 1,25-$(OH)_2$D) est augmentée.

La dose de vitamine D nécessaire à l'observation d'une toxicité chez un adulte en bonne santé n'est pas connue. Elle est variable d'un individu à l'autre[207] et dépend de facteurs environnementaux (apports calciques, cointoxication par la vitamine A, comorbidité, …) et génétiques[205], de la molécule incriminée dans le surdosage. En effet, du fait d'importantes

différences en termes de liposolubilité, d'activité et de demi-vie (3 semaines pour la vitamine D native à 15 h pour la 1,25-$(OH)_2D$)[208,209], la toxicité résultant d'une prise excessive de vitamine D native est plus prolongée dans le temps et donc de gestion plus complexe sur le plan médical que celle résultant d'une prise excessive de 1,25-$(OH)_2D$.

Sur le plan physiopathologique, l'excès d'apport exogène de 25-OH-D ou de 1,25-$(OH)_2D$ est responsable d'une surexpression du VDR responsable d'une hypersensibilité intestinale à la vitamine D [210–214], potentialisant ses effets sur l'absorption du calcium et du phosphate alimentaire. De plus, du fait d'une absence de régulation de la 25-hydroxylation, l'intoxication par la vitamine D native et la 25-OH-D conduit à de fortes élévations de la concentration sérique en 25-OH-D menant à une forte stimulation du VDR par la 25-OH-D qui entre en compétition avec la 1,25-$(OH)_2D$ pourtant plus affine pour ce récepteur[214]. Des études sur modèles murins ont également souligné le rôle de la PTH dans le contrôle de la toxicité induite par la vitamine D[215]. Le risque de toxicité induite par la vitamine D est moindre chez des souris présentant une hyperparathyroïdie secondaire à une restriction calcique comparé à celles présentant des apports calciques satisfaisant et une PTH plus basse. Cela s'explique par une plus forte stimulation de l'expression de la 1α-hydroxylase induite par la PTH, d'où une activation de la conversion de la 25-OH-D en 1,25-$(OH)_2D$, cette dernière favorisant l'expression de la 24-hydroxylase catabolisant la 25-OH-D et la 1,25-$(OH)_2D$. Ainsi, la restriction calcique dans ce contexte limite l'hypercalcémie et contribue à « fluidifier » le métabolisme de la vitamine D jusqu'à sa dégradation.

Le traitement repose sur l'éviction de la vitamine D (y compris une limitation de l'exposition aux rayonnements UV et l'utilisation d'une protection solaire chez les patients présentant une intoxication d'origine endogène), la diminution des apports calciques, l'hydratation. Des thérapeutiques complémentaires (diurétique de l'anse inhibant la réabsorption paracellulaire de calcium, glucocorticoïdes inhibant la synthèse des calbindines et favorisant l'expression de *CYP24A1*[107] et donc inhibant l'absorption intestinale [216,217] et favorisant l'excrétion urinaire de calcium[218], bisphosphonates, inhibiteurs des cytochromes P450 mitochondriaux comme le kétoconazole ou le fluconazole[219,220], chélateurs de calcium) peuvent également être employés.

Rachitismes pseudocarentiels

Ce groupe de maladies appartient au groupe des rachitismes vitaminorésistants, non améliorés par la prise de vitamine D à dose usuelle ni par l'exposition solaire. Ils sont liés à des anomalies du métabolisme de la vitamine D et doivent être distingués des anomalies du métabolisme du

phosphate (anomalie de régulation de la sécrétion de FGF23, défaut de réabsorption tubulaire de phosphate) dont les mécanismes physiopathologiques et les traitements sont différents.

Les mutations perte de fonction de *VDR* sont responsables du rachitisme pseudocarentiel de type 2 (VDDR2), une forme de rachitisme vitaminorésistant qui peut s'accompagner d'une alopécie précoce de l'enfant[14–19]. Cette maladie correspond à un défaut d'activité de la vitamine D. Elle ne sera pas traitée dans ce manuscrit.

Rachitisme pseudocarentiel de type 1B : déficit en vitamine D 25-hydroxylase

Il s'agit d'une affection décrite chez un nombre limité de patients, ce qui suggère qu'il s'agit d'une maladie extrêmement rare ou largement sous-diagnostiquée, d'autant plus que le phénotype parait plus modéré que celui du VDDR1A.

Le VDDR1B causé par les mutations perte de fonction de *CYP2R1* est caractérisé par un profil clinique et biologique évocateur de rachitisme carentiel (diagnostic différentiel principal), soit des signes cliniques et radiologiques de rachitisme et une 25-OH-D sérique basse, habituellement comprise entre 5 et 10 ng/mL (12 et 24 nmol/L)[74,77,221]. Sur le plan biologique, s'y associent une hypocalcémie, une hypophosphatémie, une élévation des phosphatases alcalines, une hyperparathyroïdie secondaire, une 1,25-$(OH)_2D$ normale mais inadaptée à l'hypocalcémie.

Les premiers patients décrits porteurs d'une mutation faux-sens perte de fonction homozygote de *CYP2R1* c.296T>C (p.Leu99Pro) (rs61495246) présentaient des signes cliniques de rachitisme apparus dans la petite enfance, entre l'âge de 2 et 7 ans, et un profil biologique évocateur avec 25-OH-D basse malgré des apports alimentaires adéquats et une 1,25-$(OH)_2D$ normale [74]. Plusieurs années plus tard, deux enfants hétérozygotes composites pour les mutations c.367+1G>A (mutation d'épissage) (rs202011621) et c.768dupT (p.Leu257Serfs*6) (*frameshift*) ont été décrits avec un phénotype d'arcature des membres inférieurs avec douleurs osseuses dans l'enfance[77]. Ces descriptions sont compatibles avec une hérédité autosomique récessive.

Néanmoins, les familles décrites par Thacher *et al.* [221] suggèrent que des mutations hétérozygotes (p.Leu99Pro et c.726A>C/p.Lys242Gln) de *CYP2R1* pourraient être responsables de formes plus modérées de rachitisme à 25-OH-D basse, voire plus tardives (âge de début des symptômes compris en 2 et 12 ans). On note chez ces patients hétérozygotes une 1,25-$(OH)_2D$ plus élevée que chez des patients homozygotes, ce qui pourrait expliquer un

phénotype plus modéré, aisément confondu avec une simple carence en vitamine D. Ainsi, ces observations posent la question d'une hérédité semi-dominante en lien avec un mécanisme de dosage génique.

Sur le plan thérapeutique, ces patients répondent à un traitement par vitamine D native à dose élevée (5000, 10 000[77], voire 50 000 UI/jour[221]) comme l'atteste l'élévation de la 25-OH-D et de la 1,25-$(OH)_2D$ sériques.

Ces observations soulignent la différence importante en termes de concentration en 25-OH-D (nmol/L) et 1,25-$(OH)_2D$ (pmol/L, soit 1000 fois moins) qui explique le maintien d'une synthèse de 1,25-$(OH)_2D$ (produit) même en cas de quantité extrêmement faible de 25-OH-D (substrat). Elles posent néanmoins la question de l'origine de cette production de 25-OH-D (activité enzymatique résiduelle liée à une perte de fonction partielle ou activité enzymatique accessoire active à des concentrations pharmacologiques de vitamine D) et de la réelle absence d'activité de la 25-OH-D dont on suppose qu'elle ne concerne que la voie génomique.

Rachitisme pseudocarentiel de type 1A : déficit en 25-hydroxyvitamine D 1α-hydroxylase

Il s'agit du rachitisme pseudocarentiel le plus fréquent, causé par les mutations bialléliques perte de fonction de *CYP27B1*[76,222–225]. Le VDDR1A est caractérisé par un tableau clinique de rachitisme débutant dans la petite enfance (avant 1 an le plus souvent) et par un profil biologique particulier avec 25-OH-D normale (excluant un rachitisme carentiel) et une 1,25-$(OH)_2D$ basse voire indétectable (<10 pg/mL). Sur le plan biologique, s'y associent également une hypocalcémie, une hypophosphatémie, une élévation des phosphatases alcalines, une hyperparathyroïdie secondaire (l'hypocalcémie stimule la sécrétion de PTH par l'intermédiaire du récepteur sensible au calcium).

Ce diagnostic ne doit pas être confondu avec un rachitisme à FGF23 élevé (hypophosphatémie liée à l'X par mutation perte de fonction de *PHEX*, rachitisme hypophosphatémique autosomique dominant par mutation gain de fonction de *FGF23* et rachitisme hypophosphatémique autosomique récessif par mutation gain de fonction de *DMP1*). En effet, il existe dans ces affections une accumulation de FGF23 responsable d'une hypophosphatémie par fuite tubulaire de phosphate mais aussi une diminution de la 1,25-$(OH)_2D$ liée à la répression de l'expression de *CYP27B1* et à une activation de l'expression de *CYP24A1*.

Un grand nombre de patients et de mutations perte de fonction sont aujourd'hui publiés, ce qui rend compte d'un diagnostic plus aisé (contrairement au déficit en 25-hydroxylase plus facilement confondu avec une carence en vitamine D) et peut être, d'une prévalence plus élevée. Le traitement du déficit enzymatique en 25-hydroxyvitamine D 1α-hydroxylase repose sur l'administration de dérivés de la vitamine D comportant une fonction hydroxyle en position 1α, soit par de la 1,25-$(OH)_2D$[226,227] ou de la 1α-OH-D_3.

Hypersensibilité à la vitamine D : déficit en vitamine D 24-hydroxylase

L'hypercalcémie est habituellement définie par une concentration en calcium sérique supérieure à la normale (2,6 à 2,7 mmol/L selon les méthodes de dosage ou 1,35 mmol/L si l'on considère sa fraction ionisée). Elle peut être aigue ou chronique, associée à une augmentation de l'excrétion urinaire de calcium (hypercalciurie). Son étiologie chez l'adulte est dominée par l'hyperparathyroïdie primaire (sécrétion inappropriée de PTH par un adénome parathyroïdien) et l'hypercalcémie paranéoplasique (sécrétion de PTHrp). Chez l'enfant, l'hypercalcémie est plus rarement observée et d'étiologie différente.

Dans les années 1950, le concept d'Hypercalcémie Infantile Idiopathique fut introduit par Lightwood et Fanconi[228,229] pour désigner une forme infantile d'hypercalcémie liée à une augmentation de l'absorption intestinale de calcium associée à une hypercalciurie[230,231]. Deux formes furent initialement distinguées : une forme syndromique dites de Fanconi qui s'avéra plus tard correspondre au syndrome de Williams Beuren (microdélétion 7q11.2[232]), une forme modérée non syndromique dite de Lightwood. Plusieurs observations permirent de relier ces cas d'hypercalcémie à un mécanisme dit d'hypersensibilité à la vitamine D : (1) une augmentation de l'incidence de cette maladie au Royaume Uni sur une période de 2 ans correspondant à une campagne de supplémentation en vitamine D à forte dose (environ 4000 UI/j), (2) un phénotype n'apparaissant que chez une minorité d'enfant recevant cette supplémentation, (3) un phénotype évocateur d'intoxication à la vitamine D malgré l'utilisation de doses de vitamine D trop faible, (4) une diminution de l'incidence de la maladie après diminution de l'enrichissement laitier en vitamine D au Royaume Uni[233]. Des observations similaires étayant cette hypothèse furent faites dans d'autres pays d'Europe[234–236].

L'observation de cas familiaux (plusieurs enfants atteints dans une même fratrie/famille), potentiellement en lien avec une consanguinité, suggérèrent une origine génétique, vraisemblablement autosomique récessive.

En 2011, deux équipes identifièrent des mutations perte de fonction dans le gène *CYP24A1* codant la vitamine D 24-hydroxylase chez des enfants présentant une Hypercalcémie Infantile Idiopathique avec néphrocalcinose et un phénotype biologique évocateur d'intoxication endogène par la vitamine D (hypercalcémie, hypercalciurie, PTH basse, 25-OH-D normale ou haute et 1,25-$(OH)_2$D élevée), la première par une stratégie de gènes candidats (séquençage de gènes impliqués dans le métabolisme de la vitamine D : *CYP27B1*, *CYP24A1*, *FGF23*, *KL*)[115], la seconde par une stratégie de séquençage d'exome[237]. Le phénotype associé à cette maladie est ainsi similaire à celui du modèle murin étudié des années auparavant[238,239]. Il fut par la suite étendu grâce à la description de patients adultes et ainsi, aux patients lithiasiques (coliques néphrétiques récurrentes de l'enfant et de l'adulte jeune)[240–249]. Un nouveau biomarqueur d'intérêt, l'élévation du ratio 25-OH-D/24,25-$(OH)_2$D sérique (>80), fut également associé à cette maladie et permet de la distinguer des intoxications à la vitamine D[250].

Il est important de souligner que le phénotype de cette maladie dépend en partie de facteurs environnementaux dont le principal est l'apport exogène de vitamine D [251]. L'exposition solaire est également responsable chez ces patients de variations des concentrations sérique et urinaire de calcium, justifiant d'une protection solaire attentive[240]. La grossesse, en particulier, du fait de l'augmentation de l'activité 1α-hydroxylase placentaire, constitue également une situation à risque d'hypercalcémie chez ces patientes, potentiellement responsable de menaces d'accouchement prématuré[252–255].

Sur le plan génétique, il s'agit d'une affection autosomique récessive, la majorité des patients décrits présentant des mutations homozygotes ou hétérozygotes composites (deux mutations hétérozygotes, chacune située sur un allèle différent). Plusieurs des variations identifiées ont pu être étudiées sur le plan fonctionnel en exprimant les mutants dans des modèles cellulaires (fibroblastes pulmonaires de hamster chinois V79-4) et en utilisant des métabolites de la vitamine D radiomarqués (**Figure 19**)[115]. Une hérédité autosomique dominante a également été suggérée[256] bien que les souris hétérozygotes ne présentent aucun phénotype, avec ou sans exposition à la vitamine D[238,239].

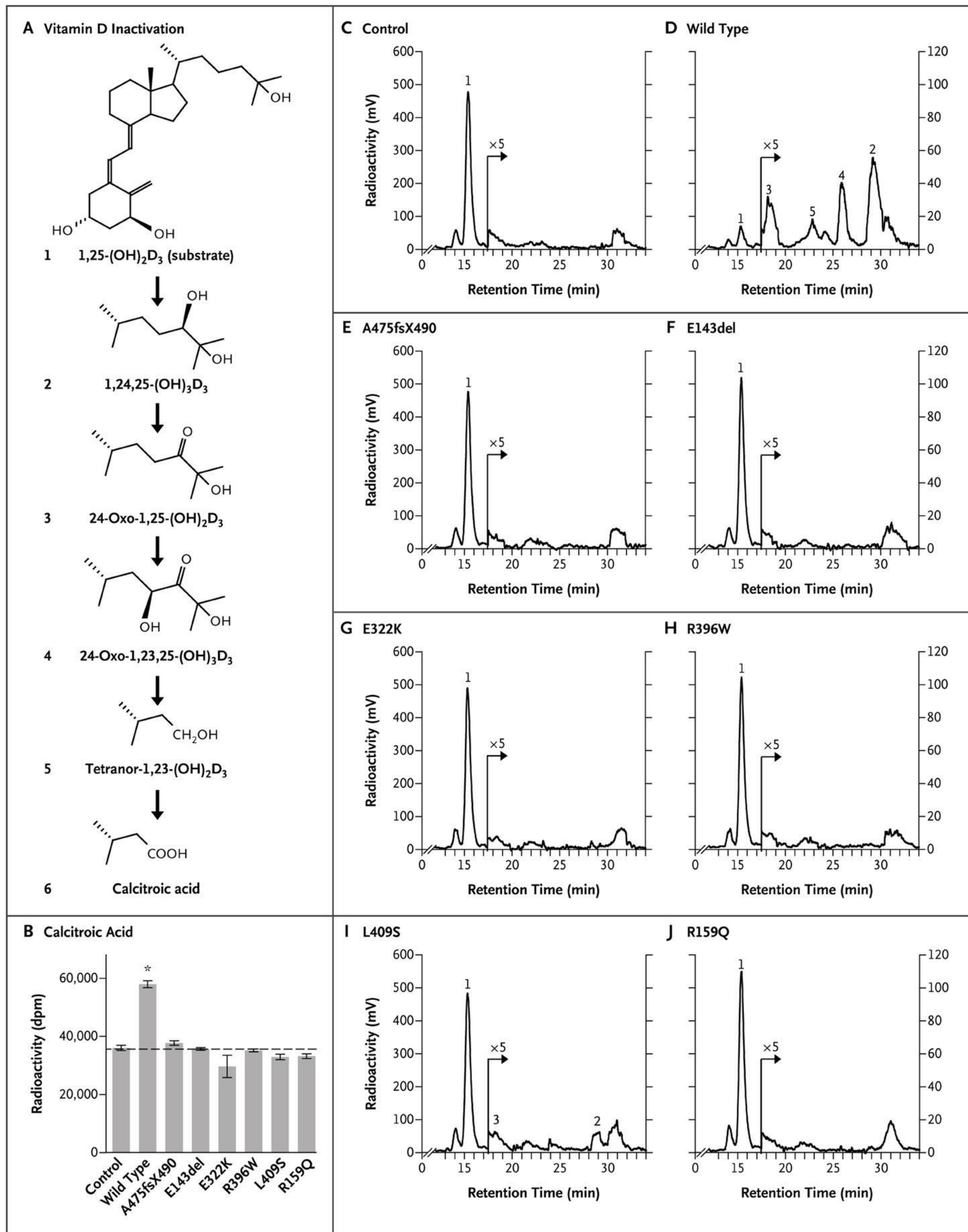

Figure 19 : Etude fonctionnelle des variants de *CYP24A1* initialement publiée par Schlingmann *et al.*

A : Produits de dégradation de la 1,25-$(OH)_2D_3$ étudiés par LC-MS/MS ; B : production finale d'acide calcitroïque ; C – J : profil LC-MS/MS des produits de dégradation des variants comparés au contrôle négatif et à l'enzyme sauvage.

En se basant sur une hérédité récessive et en utilisant les fréquences alléliques des quatre principales mutations récurrentes aujourd'hui connues (p.Glu143del, p.Leu148Pro, p.Arg396Trp et p.Leu409Ser) disponibles dans les bases de données accessibles en ligne (GnomAD), on peut estimer une fréquence des hétérozygotes à 1/140 et une fréquence de la maladie comprise entre 1/75 000 et 1/80 000.

Il n'existe pas de traitement spécifique de cette affection, en dehors de l'éviction de la vitamine D, d'une protection solaire adaptée, d'une bonne hydratation et d'une limitation des apports en calcium. Les mesures complémentaires utilisées dans le traitement de l'intoxication à la vitamine D peuvent également être utilisées, même s'il n'existe que peu de données (à court et long termes) quant à leur efficacité et leur tolérance. On notera une inefficacité relative des glucocorticoïdes chez les patients présentant un déficit en 24-hydroxylase, soulignant le rôle d'induction de l'expression de *CYP24A1* de ces molécules : si les corticoïdes permettent de stimuler l'expression d'une enzyme fonctionnelle chez les patients présentant une intoxication à la vitamine D, l'enzyme produite en cas de mutation de *CYP24A1* ne l'est pas suffisamment pour permettre une efficacité du traitement[245]. Enfin, l'utilisation des diurétiques de l'anse en pédiatrie sera prudente du fait d'un risque de majorer l'hypovolémie et donc l'hypercalcémie ainsi que le risque de néphrocalcinose ou de lithiase rénale. Les effets d'une déplétion en calcium et en vitamine D à long terme chez ces patients ne sont actuellement pas connus.

En 2016, l'étude par *homozygosity mapping* et séquençage de familles consanguines avec enfants présentant un phénotype d'hypersensibilité à la vitamine D sans mutation de *CYP24A1* ont conduit à l'identification d'un deuxième gène impliqué dans un phénotype similaire (phénocopie) : *SLC34A1* codant le cotransporteur rénal sodium-phosphate NPT2a (ou NaPiIIa)[257,258]. Il s'agit également d'une maladie autosomique récessive, bien que le rôle des mutations hétérozygotes de ce gène ne soit pas formellement établi. Le mécanisme physiopathologique suspecté dans cette affection repose sur une régulation négative du FGF23 par une fuite rénale de phosphate responsable d'une surexpression de *CYP27B1* et donc d'une dérégulation de la production rénale de 1,25-$(OH)_2D$. Cette hypothèse est soutenue par l'hypophosphatémie décrite chez ces patients, par l'efficacité de la supplémentation en phosphate pour normaliser leur bilan phosphocalcique ainsi que par l'étude d'un modèle murin $Slc34a1^{-/-}$.

De façon intéressante, un profil biologique similaire a pu être décrit chez des patients présentant des lésions génétiques dans le gène *SLC34A3* codant le cotransporteur rénal sodium-phosphate NPT2c (ou NaPiIIc). En revanche, leur phénotype clinique comporte des éléments communs mais diffère significativement de celui des patients avec mutations de *CYP24A1* ou *SLC34A1* :

ils ne présentent pas d'hypercalcémie symptomatique mais un rachitisme potentiellement associé à une néphrocalcinose ou à une lithiase rénale.

PROBLEMATIQUE GENERALE ET OBJECTIFS

Comme nous venons de le voir dans l'introduction, le métabolisme de la vitamine D encadrant la régulation de la concentration sérique de 1,25-$(OH)_2$D est placé sous le contrôle de différents facteurs extrinsèques (exposition aux UVB, apports alimentaires de vitamine D, calcium et phosphate) et intrinsèques (calcium et phosphate sériques, hormones comme la PTH ou le FGF23). L'action conjuguée de ces facteurs permet de moduler l'expression des gènes impliqués dans l'activation de la 25-OH-D en 1,25-$(OH)_2$D et le catabolisme de la vitamine D (1,25-$(OH)_2$D et 25-OH-D).

L'étude des maladies rares, dont certaines sont génétiques, a permis l'identification de séquences impliquées dans le contrôle de nombreuses voies métaboliques ou de signalisation, qu'il s'agisse de gènes ou de régions régulatrices (promoteurs) de ceux-ci. L'observation du phénotype des patients atteints de maladies rares permet de comprendre les mécanismes physiopathologiques mis en jeu dans ces maladies, mais également dans d'autres maladies touchant les mêmes voies.

Appliqué au métabolisme de la vitamine D, l'étude de patients présentant un phénotype de rachitisme avec hypocalcémie et taux anormalement bas de 1,25-$(OH)_2$D (VDDR1A) a permis l'identification de la vitamine D 1α-hydroxylase codée par le gène *CYP27B1* et de souligner le rôle de la 1,25-$(OH)_2$D en tant que forme active de la vitamine D. Plus récemment, par une approche gène candidat, des variations pathogènes perte de fonction du gène *CYP24A1* (codant la vitamine D 24-hydroxylase) ont été associées à un phénotype d'hypercalcémie par un mécanisme dit d'hypersensibilité à la vitamine D avec concentration sérique de 1,25-$(OH)_2$D inadaptée à la calcémie, soulignant le rôle majeur de cette enzyme dans le catabolisme de la vitamine D[115]. Ainsi, des variations génétiques peuvent être responsables de défauts de régulation du métabolisme de la vitamine D à l'origine de maladies.

Mon travail de thèse porte sur l'étude de patients présentant des phénotypes pathologiques (VDDR d'une part, HVD d'autre part) en lien avec des profils particuliers des concentrations sériques en 25-OH-D et 1,25-$(OH)_2$D. Le phénotype associé aux mutations perte de fonction de *CYP27B1* (VDDR1A) étant bien décrit dans la littérature médicoscientifique, notre attention s'est focalisée en amont et en aval de la 1α-hydroxylation.

Mes objectifs sont d'identifier des causes génétiques de dérégulation du métabolisme de la vitamine D et de préciser les mécanismes physiopathologiques par une description précise du phénotype.

Dans un premier temps, j'ai recherché des anomalies des gènes codant les vitamine D hydroxylases (*CYP2R1*, *CYP27B1* et *CYP24A1*, codant respectivement les 25-, 1α et 24-hydroxylases), dans un deuxième temps, des gènes codant d'autres protéines impliquées dans la biologie de la vitamine D (*GC*, *VDR*, *PDIA3* et *CYP3A4* codant respectivement la protéine de transport de la vitamine D, ses récepteurs et une enzyme 25-hydroxylase accessoire), et des gènes impliqués dans la réabsorption tubulaire de phosphate (*SLC34A1*, *SLC34A3* et *SLC9A3R1*, codant respectivement les cotransporteurs rénaux sodium-phosphate NPT2a et c et la protéine adaptatrice NHERF1). Enfin, nous avons recherché des variations des séquences régulatrices de *CYP27B1* et *CYP24A1*. Seules les séquences des promoteurs proximaux ont été étudiées, la caractérisation des séquences régulatrices distales dans le rein humain n'ayant pas été effectuée à ce jour.

MATERIEL ET METHODE

Patients

Le recrutement des patients a commencé en septembre 2012 par l'intermédiaire du Centre national de Référence Maladies rares du métabolisme du calcium et du phosphate. En accord avec la législation en vigueur, l'inclusion des patients étaient soumise à l'obtention d'un consentement du patient ou de ses représentants légaux pour l'étude des caractéristiques génétiques d'une personne à des fins de diagnostic et de recherche.

Concernant le phénotype d'hypersensibilité à la vitamine D, les critères d'inclusion comprenaient un antécédent d'hypercalcémie aigue (symptomatique ou non) ou chronique (documentation à au moins deux reprises d'une calcémie supérieure à la normale), et/ou une hypercalciurie chronique (documentation à au moins deux reprises d'une calciurie supérieure à la normale après un recueil sur 24h ou calcul d'un rapport calciurie sur créatininurie sur échantillon élevé), et/ou lithiase rénale et/ou néphrocalcinose, avec PTH adaptée soit basse ou indétectable.

Concernant le phénotype de VDDR1B, les critères d'inclusion comprenaient des signes cliniques (retard de croissance staturopondéral avec incurvation des membres inférieurs) et biologiques (élévation des PAL) de rachitisme avec 25-OH-D sérique basse ou indétectable après une supplémentation bien conduite.

Le seul critère d'exclusion considéré était l'absence de consentement.

Collection des données cliniques et biologiques

Pour chaque patient, un set minimal de données cliniques et biologiques (incluant 25-OH-D et 1,25-$(OH)_2D$, seuls métabolites habituellement étudiés dans un cadre de pratique médicale de routine en France) était demandé afin de préciser le phénotype associé à la dérégulation du métabolisme de la vitamine D.

Les dosages des métabolites de la vitamine D utilisés en routine diagnostique (25-OH-D et 1,25-$(OH)_2D$) étaient donc réalisés avec différentes techniques selon la provenance des patients, principalement par dosage immunologique direct par compétition (25-OH-D) ou radioimmunoanalyse (1,25-$(OH)_2D$), et non par chromatographie liquide couplée à la spectrométrie de masse en tandem (LC-MS/MS).

Techniques de séquençage

Deux méthodes de séquençage ont été employées : la technique classique dite Sanger et la technique de séquençage massif en parallèle (MPS ou NGS pour *next generation sequencing*) d'un panel de gènes développé au sein du laboratoire. Les deux techniques employées sont détaillées dans les publications inclues dans ce travail de thèse, respectivement A et C.

Analyse bioinformatique

L'interprétation des variations de séquence selon les critères publiés par l'*American College of Medical Genetics and Genomics* et l'*Association for Molecular Pathology* en utilisant le logiciel Alamut® (Interactive Biosoftware, Rouen, France) et la base de données Varsome (https://varsome.com - Saphetor, Lausanne, Suisse) accessible en ligne[259].

Recherche de grands réarrangements

La recherche de grands réarrangements a été faite par analyse des profils de couverture issus des données de séquençage massif en parallèle et confirmée par PCR quantitative temps réel et par séquençage des points de cassure. L'ensemble de la méthode est décrit dans la publication C.

Etudes des promoteurs proximaux de *CYP27B1* et *CYP24A1*

Les promoteurs proximaux des gènes *CYP27B1* et *CYP24A1* ont été étudiés par séquençage Sanger (méthodologie décrite dans les publications A et C). Les séquences des amorces et des références utilisées sont celles publiées par Kong *et al.*[130] et Roff et Wilson[133], respectivement.

Gène	amorce	séquence	référence
CYP27B1	F1	5′-CTT GGG AAG GTG TGG GGG T-3′	Kong et al. (adapté)
CYP27B1	R1	5′-TCC CTT GGG TCT CAT GTT GC-3′	Kong et al. (adapté)
CYP27B1	F2	5′-CTA ACC AGA AAG CCC CCC AG-3′	Kong et al. (adapté)
CYP27B1	R2	5′-ATC CGT TCT CTC TGG CTG TC-3′	Kong et al. (adapté)
CYP24A1	F	5′-GTG TCA AGG AGG GTA GAT GAG ATG-3′	Roff et Wilson 2008
CYP24A1	R	5′-TTG CTC AAG TTA AGA AAG TCT CCT C-3′	Roff et Wilson 2008

Tableau 2 : amorces utilisées pour l'amplification des promoteurs proximaux de *CYP27B1* et *CYP24A1*

Le promoteur de *CYP27B1* a été amplifié en deux fragments selon le protocole suivant :

	pour 1 tube (µL)
Enzyme Taq Eurobio	0,1
Mix 1,5 mM $MgCl_2$	23,7
Amorce sens 100 pmol/L	0,1
Amorce antisens 100 pmol/L	0,1
ADN 120 ng/µL	1

	pour 48 µL
Tampon 10X	5
$MgCl_2$ (25mM)	3
dNTP (10 mM)	1
H2O	39

Tableau 3 : Mélange réactionnel pour l'amplification du promoteur de *CYP27B1*

Compte tenu d'une richesse en GC de 65 %, l'amplification du promoteur proximal de *CYP24A1* a été réalisée avec le kit *TaKaRa LA Taq ® with GC Buffer* (Takara Bio Inc, Shiga, Japon) selon le protocole suivant :

	Pour 1 tube (µL)
Enzyme Takara LA Taq	0,25
Tampon 2X GC Buffer I	12,5
dNTP mixture (2,5mM de chaque)	4
Amorce sens 100pmol/L	0,1
Amorce antisens 100pmol/L	0,1
H_2O	7,05
ADN 120ng/µL	1

Tableau 4 : Mélange réactionnel pour l'amplification du promoteur de *CYP24A1*

Cycle d'amplification :

5mn à 95°C (hot start) → (20s à 95°C ; 20s à 65°C -0.5°C/cycle ; 45s à 72°C) x10 cycles (*touchdown*) → (20s à 95°C ; 20s à 60°C ; 45s à 72°C) x32 cycles → 10min à 72°C, retour à 10°C

Analyses de fonctionnalités

La pathogénicité des variations identifiées dans les gènes *CYP2R1* et *CYP24A1* a été analysée par étude de fonctionnalité *in vitro* selon la méthodologie décrite dans les publications A et B.

Analyse biochimique par chromatographie liquide couplée à la spectrométrie de masse en tandem

Le dosage de la 24,25-$(OH)_2D$ et l'évaluation du rapport sérique 25-OH-D / 24,25-$(OH)_2D$ a été réalisé par LC-MS/MS selon la méthodologie décrite dans les publications A et c.

RESULTATS

Patients

Au total, 2 familles avec phénotype de VDDR à 25-OH-D basse et 200 échantillons de patients présentant un phénotype HVD ont été étudiés sur le plan génétique. Un résumé détaillé de la présentation clinique et biologique de ces patients est présenté dans les publications B et A/D.

Trois patients porteurs de mutations du gène *CYP27B1* dans le cadre d'un VDDR1A ont également été étudiés sur le plan biochimique (évaluation du rapport 25-OH-D / 24,25-$(OH)_2D$ par LC-MS/MS).

Etude de *CYP2R1*

Il n'a pas été identifié de variation pathogène de *CYP2R1* chez des patients avec phénotype HVD.

En revanche, des variations pathogènes de ce gène ont été identifiées dans deux familles avec VDDR1B. Compte-tenu du petit nombre de cas publiés et des divergences entre les patients et le modèle murin, il semblait opportun de préciser le phénotype, en particulier biochimique, dans ces familles.

Le rachitisme pseudocarentiel de type IB (déficit en 25-hydroxylase) : une maladie rare ou sous-diagnostiquée ?

Vitamin D-Dependent Rickets Type 1B (25-Hydroxylase Deficiency): A Rare Condition or a Misdiagnosed Condition?

Cet article paru en 2017 dans *Journal of Bone and Mineral Research* porte sur sept individus issus de deux familles avec déficit en vitamine D 25-hydroxylase confirmé par l'identification de deux variations pathogènes de *CYP2R1*, une nouvelle variation c.124_138delinsCGG (p.Gly42_Leu46delinsArg) et la variation c.296T>C (p.Leu99Pro) publiée en 2004, toutes deux caractérisées sur le plan fonctionnel. Ce travail souligne le chevauchement phénotypique qui existe entre le déficit en vitamine D 25-hydroxylase et le rachitisme par carence en vitamine D, du fait d'un profil vitaminique (25-OH-D basse, 1,25-$(OH)_2D$ normale ou élevée, 24,25-$(OH)_2D$ normale sous traitement), d'une évolution en trois phases et d'une présentation radiologique superposables. Ces éléments expliquent vraisemblablement le peu de cas identifiés, diagnostiqués à tort comme des rachitismes carentiels, d'autant que les patients adultes semblent asymptomatiques. Une étude ostéodensitométrique a révélé que ces patients adultes ne présentent pas d'ostéoporose (communication affichée présentée lors du 35ème Congrès de la Société Française d'Endocrinologie).

Avant l'établissement du diagnostic moléculaire, le traitement de l'un de ces patients par alfacalcidol (Un Alfa® ou 1α-hydroxyvitamine D_3) a permis une amélioration partielle du phénotype avec augmentation majeure de la 1,25-$(OH)_2D$ suggérant le recrutement de 25-hydroxylase(s) accessoire(s). Enfin, dans un concept de médecine personnalisée, cette étude apporte la preuve de l'efficacité d'un traitement quotidien *per os* de cette affection par Dédrogyl® (calcifédiol ou 25-OH-D_3).

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Vitamin D–Dependent Rickets Type 1B (25-Hydroxylase Deficiency): A Rare Condition or a Misdiagnosed Condition?

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ABSTRACT

Vitamin D requires a two-step activation by hydroxylation: The first step is catalyzed by hepatic 25-hydroxylase (*CYP2R1*, 11p15.2) and the second one is catalyzed by renal 1α-hydroxylase (*CYP27B1*, 12q13.1), which produces the active hormonal form of 1,25-$(OH)_2$D. Mutations of *CYP2R1* have been associated with vitamin D–dependent rickets type 1B (VDDR1B), a very rare condition that has only been reported to affect 4 families to date. We describe 7 patients from 2 unrelated families who presented with homozygous loss-of-function mutations of *CYP2R1*. Heterozygous mutations were present in their normal parents. We identified a new c.124_138delinsCGG (p.Gly42_Leu46delinsArg) variation and the previously published c.296T>C (p.Leu99Pro) mutation. Functional in vitro studies confirmed loss-of-function enzymatic activity in both cases. We discuss the difficulties in establishing the correct diagnosis and the specific biochemical pattern, namely, very low 25-OH-D suggestive of classical vitamin D deficiency, in the face of normal/high concentrations of 1,25-$(OH)_2$D. Siblings exhibited the three stages of rickets based on biochemical and radiographic findings. Interestingly, adult patients were able to maintain normal mineral metabolism without vitamin D supplementation. One index case presented with a partial improvement with 1alfa-hydroxyvitamin D_3 or alfacalcidol (1α-OH-D_3) treatment, and we observed a dramatic increase in the 1,25-$(OH)_2$D serum concentration, which indicated the role of accessory 25-hydroxylase enzymes. Lastly, in patients who received calcifediol (25-OH-D_3), we documented normal 24-hydroxylase activity (*CYP24A1*). For the first time, and according to the concept of personalized medicine, we demonstrate dramatic improvements in patients who were given 25-OH-D therapy (clinical symptoms, biochemical data, and bone densitometry). In conclusion, the current study further expands the *CYP2R1* mutation spectrum. We note that VDDR1B could be easily mistaken for classical vitamin D deficiency. © 2017 American Society for Bone and Mineral Research.

KEY WORDS: RICKETS; VITAMIN D; 25-HYDROXYLASE; GENETICS; PERSONALIZED MEDICINE

Received in original form February 1, 2017; revised form May 22, 2017; accepted May 25, 2017. Accepted manuscript online May 26, 2017.
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Additional Supporting Information may be found in the online version of this article.

Journal of Bone and Mineral Research, Vol. 32, No. 9, September 2017, pp 1893–1899
DOI: 10.1002/jbmr.3181
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Introduction

Vitamin D requires a two-step activation by hydroxylation: The first step is catalyzed by 25-hydroxylase (*CYP2R1*, 11p15.2) and the second one is catalyzed by 1α-hydroxylase (*CYP27B1*, 12q13.1), which produces the active hormonal form of 1,25-$(OH)_2D$. The major function of vitamin D is to maintain serum calcium and phosphate levels within the normal physiological range to support most metabolic functions, neuromuscular transmission, and bone mineralization. An inadequate calcium-phosphate product leads to a bone mineralization defect that causes rickets in children and osteomalacia in adults. Classical vitamin D deficiency is the most common cause of rickets and has been used to justify public health recommendations for vitamin D supplementation in children.

Rickets with adequate dietary intake or sunlight exposure has been associated with rare hereditary disorders. Defects in 25- or 1α-hydroxylation lead to an inability to activate vitamin D, thus causing a vitamin D deficiency–like state that is resistant to physiologic doses of vitamin D. Vitamin D–dependent rickets type 1A (VDDR1A, MIM 264700) was first described and associated with loss-of-function mutations of *CYP27B1*. Mutations of *CYP2R1* have recently been associated with VDDR1B (MIM 600081), a very rare condition that has been reported to currently affect only 4 families.[1–5]

In this article, we described the molecular analysis of 7 patients from 2 unrelated families who presented with VDDR1B due to a loss-of-function mutation of *CYP2R1* and a new mutation. We noted the specific biochemical pattern of the disease and its dramatic improvement with 25-hydroxyvitamin D therapy.

Patients and Methods

In family 1 (F1) (Fig. 1*A*), the proband (II-3) is a 4-year-old boy, the third child of a non-consanguineous healthy French couple. He received daily vitamin D_3 supplementation according to French recommendations (1000 to 1200 IU/d or 25 to 30 μg/d of cholecalciferol) until the age of 18 months. He received medical attention for growth restriction (height, –4 SD; weight, –2 SD) with lower limb abnormalities (*genu varum*) and mild hypotonia. Biochemical analyses were suggestive of vitamin D deficiency and nutritional rickets, as revealed in the lower limb radiography images (Fig. 2). This patient was first diagnosed with 1α-hydroxylase deficiency and was treated with calcium supplementation and alfacalcidol (1α-OH-D_3). Once the exact diagnosis of VDDR1B was achieved, he was subsequently treated with calcifediol (25-OH-D_3).

In family 2 (F2) (Fig. 1*B*), the proband (II-1) is a 9-year-old boy, born at 40 gestational weeks (birth weight 3370 g, birth length 49 cm, birth head circumference 35 cm, Apgar 9/10), to consanguineous healthy parents from Moroccan extraction. Cognitive and motor skill development were normal except for a walking delay (23 months). Medical history began at the age of 5 years with the exploration of a waddling gait. He was supplemented with vitamin D during infancy (800 IU/d of cholecalciferol until age 18 months); later he received a yearly bolus of vitamin D (100,000 IU of cholecalciferol every autumn). At 7 years old, he complained of muscular pain and increased fatigability. He presented with proximal muscular deficiency and dramatic amyotrophy of the lower limbs suggestive of myopathy, but a muscular biopsy was normal. Finally, the diagnosis of VDDR1B rickets was suspected because of radiological (Fig. 2) and biological data (Table 1).

Written informed consent was obtained from the patients and/or their parents for the collection of clinical and laboratory data and for genetic investigation. The molecular analysis of relatives was suggested through genetic counseling after the identification of a mutation in the probands.

Biochemical investigations (Tables 1 and 2)

Routine laboratory data were collected at the time of the diagnosis, retrospectively and prospectively, using records from hospitals. Intact parathyroid hormone (PTH 1-84), 1,25-$(OH)_2D$ and alkaline phosphatase (ALP) were quantified in the serum via a chemiluminescent immunoassay (LIAISON XL Analyzer, DiaSorin, Stillwater, MN, USA). 25-OH-D levels were assessed via the following two methods: a chemiluminescent immunoassay for the quantitative determination of total 25-OH-D and a Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) assay for the simultaneous quantification of 25-OH-D_2, 25-OH-D_3, and 24,25-$(OH)_2D_3$. CYP24A1 (24-hydroxylase) enzyme activity was quantified by LC-MS/MS from the ratio of 25-OH-D_3: 24,25-$(OH)_2D_3$ as previously described.[6]

The analytical performance of the various methods is described in the supplemental material.

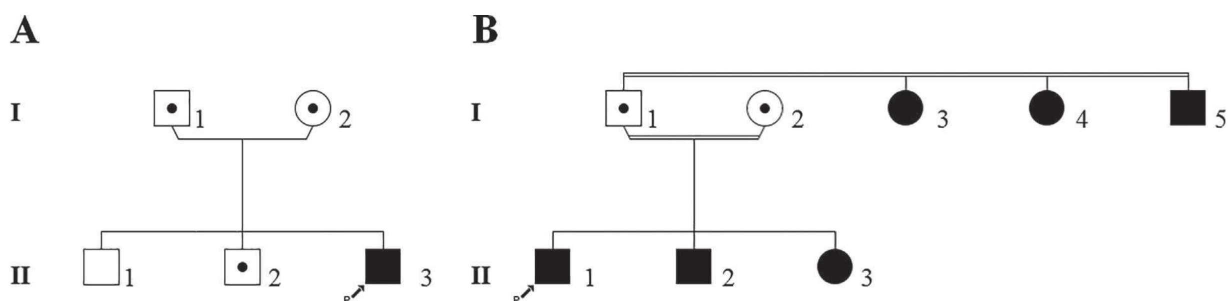

Fig. 1. (*A*) Pedigree tree of family 1 with the *CYP2R1* mutation c.124_138delinsCGG (p.Gly42_Leu46delinsArg). (*B*) Pedigree tree of family 2 with the *CYP2R1* mutation c.296T>C (p.Leu99Pro). ⊡ = heterozygous carrier; ■ = affected individuals; □ = unaffected individual without mutation; ═ = consanguinity; P = proband.

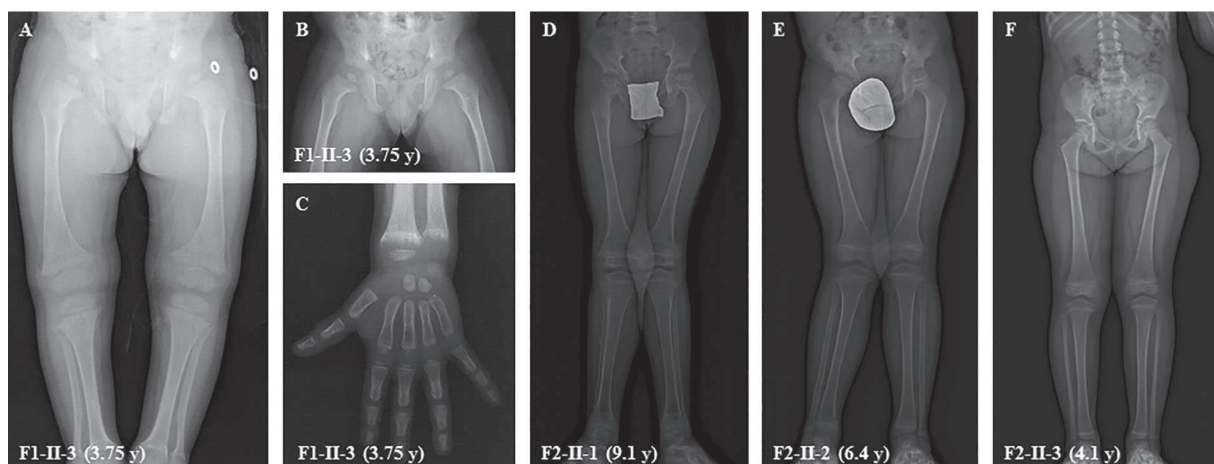

Fig. 2. Radiological features of rickets in VDDR1B patients with classical cupping and fraying of the metaphyseal region and bowing of the legs. Proband in family 1 (*A–C*) at 3.75 years. Proband in family 2 (*D*) and siblings (*E*, *F*) at the ages of 9.1, 6.4, and 4.1 years, respectively. Of note, patient F2-II-3 showed only very moderate metaphyseal dysplasia without bowing of the lower limbs.

Table 1. Biochemical Data and Clinical Evolution

		Routine serum assay					
		Ca	P	PTH (1-84)	ALP	25-OH-D	1,25-(OH)$_2$D
Patient age		mmol/L	mmol/L	pg/mL	UI/L	nmol/L	pmol/L
Normal range	Therapy	2.20–2.65	*	6.5–36.8	—	62.4–282	20–100
F1-II-3							
3.75 yr	D$_3$ 25–30 μg/d	1.57	1.44	216	762	<10	194
3.8 yr	Ca 500 mg/d and 1α-OH-D$_3$ 4 μg/d (after 1.5 m)	2.55	2.1	96.9	513	10	383
4.5 yr	25-OH-D$_3$ 20 μg/d during 0.5 mo (after 2 mo)	2.6	1.6	14.7	302	268	221
5.5 yr	25-OH-D3 20 μg/d	2.48	1.84	21.7	218	182	187
F1-I-1 35 yr		2.3	0.8	29.2	71	59	179
F1-I-2 33.5 yr		2.4	1.3	24.9	62	38	150
F1-II-1 9.5 yr		2.45	1.71	22.5	256	60	157
F1-II-2 7 yr		2.43	1.74	16	232	39	165
F2-II-1							
8.75 yr		1.98	1.07	338	1921	<10	36
9.1 yr	Ca 500 mg/d and 25-OH-D$_3$ 15 μg/d (after 3 mo)	2.27	1.74	59.9	595	164	731
9.75 yr	Ca 500 mg/d and 25-OH-D$_3$ 15 μg/d (after 1 yr)	2.5	1.58	33.1	374	211	372
F2-II-2							
6.2 yr		1.38	1.29	345	581	<10	64
6.4 yr	Ca 500 mg/d and 25-OH-D$_3$ 15 μg/d (after 3 mo)	2.48	1.78	10.2	320	250	285
7.1 yr	Ca 500 mg/d and 25-OH-D$_3$ 15 μg/d (after 1 yr)	2.48	1.65	23.4	394	218	235
F2-II-3							
3.8 yr		2.45	1.42	130	266	<10	117
4.1 yr	Ca 500 mg/d and 25-OH-D$_3$ 15 μg/d (after 3 mo)	2.65	1.45	6.3	298	410	189
4.8 yr	Ca 500 mg/d and 25-OH-D$_3$ 15 μg/d (after 1 yr)	2.45	1.36	24	294	480	214
F2-I-3							
17.75 yr	Ca 4.5 g/d and 25-OH-D$_3$ 50 μg/d (after 1 yr)	2.28	0.78	37		153	68
20.9 yr	Ca 1.5 g/d and 25-OH-D$_3$ 15 μg/d: bad compliance	2.3	0.9	39	128	<10	
F2-I-4							
11.6 yr	No treatment	2.3	0.7	375	3762	<10	83
11.7 yr	Ca 1 g/d and 25-OH-D$_3$ 50 μg/d (after 1 mo)	2.5	1.4	59	1400	102.3	
16.5 yr	Ca 1 g/d and 25-OH-D$_3$ 50 μg/d: bad compliance	2.2	1.3	109	255	<10	122.4
31.8 yr	No treatment	2.5			233		
F2-I-5							
11.8 yr	No treatment	2.1	0.7		1487	<10	83
12.25 yr	Ca 2 g/d and 25-OH-D$_3$ 40 μg/d (after 3 mo)	2.6	1.6	14	551	185	145
30.7 y	No treatment	2.3		50		<10	208

Ca = calcium; yr = year(s); mo = month(s); d = day(s).

*Normal values for serum phosphate (P) according to age: 1–5 years: 1.3–2.4 mmol/L; 5–13 years: 1.3–1.9 mmol/L; >13 years: 0.9–1.8 mmol/L.

Table 2. Vitamin D Metabolites Identified Via LC-MS/MS

		LC-MS/MS assay			
		25-OH-D_3	25-OH-D_2	$24,25\text{-(OH)}_2\ D_3$	Ratio
	Vitamin D therapy	nmol/L	nmol/L	nmol/L	$N < 25$
F1-II-3 4.7 yr	25-OH-D_3 20 μg/d	309.3	1.2	28.6	10.8
F2-II-1 9.1 yr	25-OH-D_3 15 μg/d	204.9	1.0	13.2	15.5
F2-II-2 6.4 yr	25-OH-D_3 15 μg/d	302.3	1.2	22.6	13.4
F2-II-3 4.1 yr	25-OH-D_3 15 μg/d	460.3	1.5	54.2	8.5
F2-I-5 30.7 yr	No treatment	6.7	0.2	0.2	33.5

Normal 25-OH-D_3: $24,25\text{-(OH)}_2\ D_3$ ratio in children receiving oral 25-OH-D_3 as a result of a normal 24-hydroxylase activity. Of note, patient F2-I-5 has a low but detectable 25-OH-D_3.

Bone densitometry

Bone densitometry was assessed in the lumbar spine and total hip with a QDR 4500 A Hologic device (Hologic, Stefanix, France). The results are given as *Z*-scores. The analytical performance of the method is described in the Supplemental Material.

CYP2R1 molecular analysis

Genomic DNA was isolated from peripheral blood leukocytes via standard methods. We designed several sets of primers for the amplification and sequencing of *CYP2R1* exons 1–5 and their intron-exon junctions (available on request). PCR products were purified and sequenced with the CEQTMDTCS Quick Start Kit (Beckman Coulter, Brea, CA, USA) on a Beckman Coulter DNA Sequencer.

Genetic variations were studied in silico with prediction tools (Polyphen-2, Align GVGD, Mutation Taster, SIFT) and data frequencies from Exome Variant Server (EVS) and Exome Aggregation Consortium (EXAC) databases. Data on the functional activity of each variant were searched in published literature. The OPM server (Orientations of Proteins in Membranes; http://opm.phar.umich.edu/server.php) was used to determine the position of variants in vitamin D 25-hydroxylase and its consequences on membrane binding.

In vitro functional analysis of CYP2R1 variants

A *CYP2R1* mutation was introduced into the wild-type hCYP2R1 with QuikChange (Stratagene Corp., La Jolla, CA, USA), according to the manufacturer's protocol and oligonucleotide pair bases specific for each variation (Integrated DNA Technologies, Inc., Coralville, IA, USA). Mutations were confirmed by sequencing (Robarts Research Institute, London, Canada), and the plasmids used for transfection were purified with QIAprep Spin Miniprep Kit (Qiagen Inc., Mississauga, Canada).

Human wild-type and mutated *CYP2R1* constructs were transiently transfected into V79-4 Chinese hamster lung fibroblast cells. These cells have no *CYP2R1* basal activity and are a favorite host system for a variety of CYPs. Transfected cells were incubated with 500 nM $1\alpha\text{-OH-D}_2$, and the incubation media was extracted via the Bligh and Dyer method. Enzyme extracts were immunopurified over a column containing anti-$1,25\text{-(OH)}_2\text{D}$ antibodies anchored to a resin. The aqueous phase was re-extracted with 0.1% glacial acetic acid and 2.5 mL of methylene chloride and dried. The samples were then derivatized by redissolving the dry residue in DMEQ-TAD as previously described.[6] The derivatized extract was dried and redissolved in 50 μL of 60:40 (vol/vol) methanol/water running solvent, and LC-MS/MS analysis was performed.[6] The $1,25\text{-(OH)}_2\text{D}_2$ product was quantitated by LC-MS/MS using a deuterated internal standard for $1,25\text{-(OH)}_2\text{D}_2$. The DMEQ-TAD adduct peaks exactly correspond to $1,25\text{-(OH)}_2\text{D}_2$ at 12.5 and 13.4 minutes. The amount of CYP2R1 products that included $1,25\text{-(OH)}_2\text{D}_2$ from $1\alpha\text{-OH-D}_2$ were used to compare the relative activities of variants to wild-type CYP2R1. The transient transfections were performed twice, each time with triplicate evaluations of each mutant and wild-type CYP2R1.

Results

Biochemical data are reported in Table 1.

At the time of diagnosis, the two probands presented with marked hypocalcemia, hypophosphatemia, high parathyroid hormone (PTH) and alkaline phosphatase (ALP) levels; undetectable 25-OH-D concentrations and high $1,25\text{-(OH)}_2\text{D}$ values led to the diagnosis of vitamin D deficiency, and they were treated accordingly.

Patient F1-II-3 was initially treated with calcium and oral alfacalcidol ($1\alpha\text{-OH-D}_3$) that led to a relief of symptoms and normalized serum calcium and phosphate but not PTH and ALP. After 9 months with this therapy, hypercalcemia with hypercalciuria was observed and calcium supplementation was stopped. Once the final diagnosis was achieved, he was subsequently treated with calcifediol (25-OH-D_3). This treatment resulted in the prompt normalization of all biochemical parameters (including PTH and ALP) and the correction of long bone deformations. Biochemical parameters in his parents and brothers were within the normal ranges. We documented normal 24-hydroxylase activity when patients were given 25-OH-D_3.

In family 2, patient F2-II-2 (the proband's brother) exhibited *genu varum*, and presented the same biochemical pattern at the age of 6.2 years. Conversely, the sister (F2-II-3) was asymptomatic and had normal serum calcium and ALP but increased PTH and low/normal phosphate at the age of 3.8. She exhibited mild metaphyseal dysplasia at the age of 4.1 years.

An initial bone densitometry exploration showed low vertebral bone density in the 3 children. A dramatic amelioration of clinical and biochemical phenotypes and a major improvement in vertebral bone density were observed with 25-OH-D_3 treatment (Table 3). However, despite good compliance to the therapy, patient F2-II-2's *genu varum* was treated by epiphysiodesis at the age of 10 years.

Patients F2-I-3, F2-I-4, and F2-I-5 are the paternal aunts and uncle of the 3 children, also born to consanguineous parents.

Table 3. Bone Densitometry in Family 2, Proband F2-II-1, and Siblings F2-II-2 and F2-II-3, Before and After 6 Months of 25-OH-D_3 Treatment

	F2-II-1		F2-II-2		F2-II-3	
Age (years)	8.8	9.4	6.2	6.8	3.8	4.4
Z-score						
Femoral neck	−3.6	0.2	−1.8	1.5	—	—
Lumbar spine	−2.2	0.5	−1.2	1.2	−0.5	0.7
Whole body	−3.1	0.1	−0.5	1.8	1.5	2

They were followed during childhood for so-called "severe nutritional rickets" and were given 25-OH-D_3. Variable compliance over time of patients F2-I-3 and F2-I-4, confirmed by regularly undetectable levels of 25-OH-D, caused recurrent complaints of bone pain, back pain, paresthesias, and headaches. Patient F2-I-3 underwent epiphysiodesis. Conversely, patient F2-I-5 was given calcium 1500 mg/d and 25-OH-D_3 15 μg/d that corrected the lower limb deformity. He remained asymptomatic and reached a height of 170 cm. At the time of diagnosis, they were 20.9, 31.8, and 32.8 years old, respectively. Serum concentrations of calcium, PTH, and ALP were within the normal range. However, routine biochemical investigations showed an undetectable concentration of 25-OH-D with a normal/high 1,25-$(OH)_2D$.

The molecular analysis of *CYP2R1* identified a variation c.124_138delinsCGG (p.Gly42_Leu46delinsArg) and the mutation c.296T>C (p.Leu99Pro) in a homozygous state in both probands of family 1 and 2, respectively. Heterozygous mutations were present in their normal parents.

The p.Leu99Pro variant was previously described by Cheng and colleagues.[2] The p.Gly42_Leu46delinsArg variant was not reported in a study of 200 control patients. In silico studies showed that the protein lacked one of its fourth intramembranous hydrophobic domains, suggesting a modification of the three-dimensional conformation of the substrate accessing channel. Finally, in vitro studies found no activity over that in mock-transfected cells of both mutants compared with wild type (Fig. 3).

Discussion

This study underlines the misdiagnosis observed in patients with VDDR1B who exhibit normal/high 1,25-$(OH)_2D$ with low/undetectable 25-OH-D levels and the dramatic improvement when patients were given 25-OH-D_3. It provides evidence for the role of this enzyme in vitamin D activation in humans. The

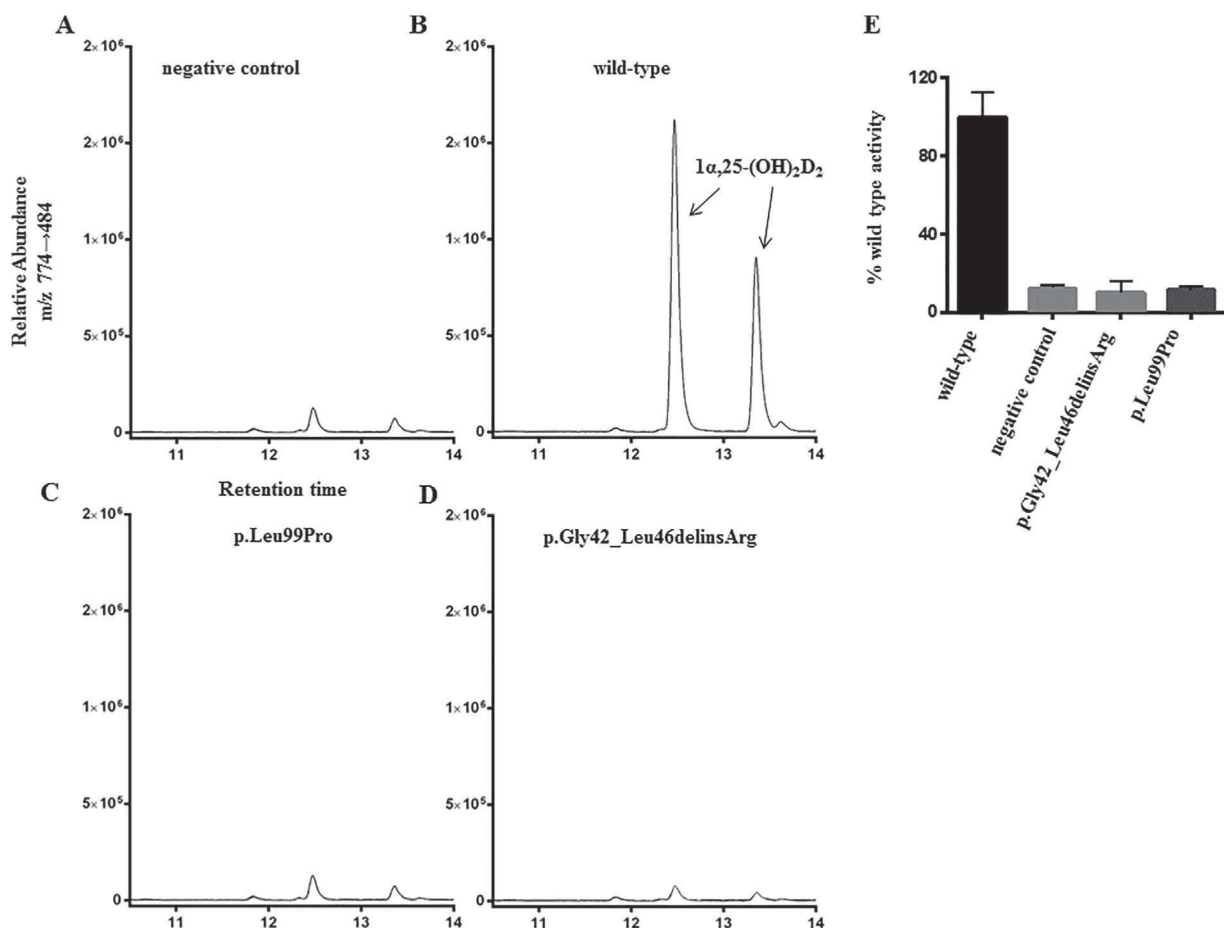

Fig. 3. Functional analysis of *CYP2R1* mutations. (*A–D*) Chromatographs showing deficient vitamin D 25-hydroxylase activity. (*E*) Histogram showing 25-hydroxylase enzymatic activities of the CYP2R1 variant.

scarcity of 25-hydroxylase deficiency cases may reflect a misdiagnosis, as many subjects with hereditary defects in vitamin D_3 metabolism receive dietary supplementation that results in normal levels of 1,25-$(OH)_2D$. Patients with VDDR1B carry mutations in a homozygous state. Relatives of these patients carry mutations in a heterozygous state, and in family 1, presented with normal clinical and laboratory parameters, according to a classic recessive autosomal inheritance.

To date, 4 *CYP2R1* mutations were characterized in vitro as 25-hydroxylase loss-of-function mutations: c.296T>C (p.Leu99Pro),[2] c.726A>C (p.Lys242Asn),[7] c.768dup (p.Leu257Serfs*6), and a splice mutation in the donor sequence of exon 2 c.367+1G>A.[5]

The c.296T>C (p.Leu99Pro) variation (rs61495246) was initially described in two unrelated Nigerian families[1] and was characterized as a loss-of-function mutation that was responsible for vitamin D_3 hydroxylase deficiency.[2] Its allelic frequency is approximately 0.46% in the African American population in EVS and 0.40% in the EXAC African population, suggesting a founder effect. However, linkage analysis that was performed by genotyping siblings of family 2 for single nucleotide polymorphisms ruled out any association with this population (data not shown). The c.124_138delinsCGG (p.Gly42_Leu46delinsArg) variation found in family 1 has never been described and was confirmed in vitro as a loss-of-function mutation.

Patients described herein exhibited a clinical history and classical features suggestive of rickets, and an initial laboratory analysis showed very low 25-OH-D, suggestive of classical vitamin D deficiency. These siblings reminded us of the evolving stages of rickets previously described in nutritional rickets, with considerable overlap[8]; they reflected the initiation of calcium homeostasis, followed by skeletal calcium depletion and increased vitamin D requirements for growth. The first stage, usually undiagnosed, is characterized by the establishment of chronic hypocalcemia, eventually marked by non-specific signs of hypocalcemia but without any bone lesions; the second stage is marked by secondary hyperparathyroidism to correct the serum calcium level, which leads to decreased serum phosphate and an elevation of ALP, osteomalacia, and the progressive apparition of skeletal signs of rickets (patient F2-II-3). The third stage is characterized by marked clinical features of rickets and symptomatic hypocalcemia, as the homeostatic mechanisms are no longer effective.

The finding that serum levels of 25-OH-D remain low despite the administration of supraphysiological doses of vitamin D without evidence of malabsorption suggested the diagnosis of VDDR1B. In this context, the normal concentration of 1,25-$(OH)_2D$, which was also described in other VDDR1B[1,3–5] patients, appeared particularly intriguing. Serum 1,25-$(OH)_2D$ concentrations have been reported to be low, normal, or even elevated in patients with vitamin D deficiency.[9–12] However, these levels of 1,25-$(OH)_2D$ could be considered inappropriately low for the degree of hyperparathyroidism, incapable of maintaining normal calcium homeostasis, and variable over time, depending on the different stages of the disease.[13,14] Moreover, patient F1-II-3 exhibited a dramatic increase in 1,25-$(OH)_2D$ when they received 1α-OH-D_3, suggesting the presence of 25-hydroxylase activity. Similarly, previously described patients responded to treatment with large doses of vitamin D and did have measurable levels of 1,25-$(OH)_2D_3$.[1–3] Because 1α-OH-D_3 is known to be a good substrate for all 25-hydroxylases (eg, CYP2R1 and CYP27A1) and circulating 1,25-$(OH)_2D$ is required only in the pmol/L range, the level of 1,25-$(OH)_2D$ observed in our patients could be a product of a mutant CYP2R1 with residual activity or a product of alternative 25-hydroxylases as postulated previously. Similarly, 1,25-$(OH)_2D$ detected in vitamin D_3–treated patients could be the product of small amounts of 25-OH-D_3 generated by defective CYP2R1 or from alternative 25-hydroxylases. Subsequent 1α-hydroxylation of the small amounts of 25-OH-D_3 that were formed would then be due to the massive stimulation of *CYP27B1* expression by high PTH levels, as observed in patient F1-II-3. Residual 25-hydroxylase activity for vitamin D_2 of p.Leu99Pro could result in 25-OH-D synthesis as previously suggested,[2] but the administration of supra physiological doses of vitamin D had a negligible effect on the serum level of 25-OH-D.[4,5] The presence of alternative vitamin D 25-hydroxylases is, therefore, likely to be the most plausible explanation for the 1,25-$(OH)_2D_3$ that was detected.[15]

Unlike CYP27B1, the unique vitamin D 1α-hydroxylase, several other cytochrome P450 enzymes have been suggested to have vitamin D 25-hydroxylase activity in a variety of biochemical and clinical studies.[16,17] Recent studies involving the murine knockout model Cyp2r1$^{-/-}$ and Cyp2r1$^{-/-}$/Cyp27a1$^{-/-}$ double knockout mice both showed similar circulating levels of 25-OH-D_3, which were 50% lower than in wild-type mice.[18] Similar mechanisms could be involved in humans.[19] CYP27A1, a mitochondrial liver sterol 27-hydroxylase, can contribute to the formation of 25-OH-D from cholecalciferol (vitamin D_3) but not from ergosterol (vitamin D_2). CYP2J2, the arachidonic acid epoxygenase, has preferential 25-hydroxylase activity toward vitamin D_2 over vitamin D_3. CYP3A4, another cytochrome P450 microsomal hepatic enzyme, activates 1α-OH-D_3 to form 1,25-$(OH)_2D$.[16,17,19,20] Although these alternate enzymes may contribute to the detectable levels of serum 25-OH-D_3, they are unable to generate the higher nanomolar serum concentrations that are usually observed in normal patients. On the other hand, these enzymes could be involved and even up-regulated, testifying to an adaptation in CYP2R1-deficient subjects to the need in active vitamin D. Consequently, the amounts of 25-OH-D_3 produced would be insufficient to satisfy the demands for growth and bone formation in order to prevent rickets during childhood. This accessory activity could also explain a later onset of VDDR1B compared with VDDR1A. Indeed, our clinical findings were similar to the Saudi and Nigerians cases, in which clinical symptoms appeared later (usually after 2 years) than in VDDR1A.[15] Accordingly, patient F2-II-3 was still asymptomatic at the age of 4 years.

Although a high-dose cholecalciferol[3,5] or 1α-OH-D_3 administration resulted in a dramatic increase in 1,25-$(OH)_2D$ and normal or even high calcium levels and an improvement in deformities, the biochemical pattern was not fully normalized, as PTH and ALP levels remained above the normal values. These observations suggest that 1,25-$(OH)_2D_3$ is not the only biologically active metabolite of vitamin D, as was previously suggested.[21] Treatment of CYP2R1-deficient patients with 1,25-$(OH)_2D_3$ was not considered because of the risks of hypercalciuria and nephrocalcinosis.[22] Consistent with a concept of personalized medicine, we showed that a replacement therapy adjusted to VDDR1B physiopathology must be based on bypassing the enzymatic defect. Calcifediol (25-OH-D_3) takes advantage of the high PTH-induced *CYP27B1* expression and leads to a global improvement in clinical and laboratory data. Patient F2-II-3, who was treated before the clinical symptoms appeared, did not present any bone deformations.

Interestingly, adult subjects in family 2 are able to sustain near-normal serum levels of calcium, PTH, and ALP despite homozygosity for the non-functional p.Leu99Pro allele. This feature was also described in another patient with VDDR1B[4] and confirmed the need for 25-OH-D_3 or an active vitamin D during childhood.

Finally, in our study, we detected normal 24-hydroxylase activity (*CYP24A1*) in patients receiving 25-OH-D_3 with normal values for the 25-OH-D_3: 24,25-$(OH)_2D_3$ ratio (Table 2).[6] This 24-hydroxylase activity is very low when serum 25-OH-D_3 levels are below 25 nmol/L,[6] likely repressed by high PTH levels,[1,23] and very little or no 24,25-$(OH)_2D_3$ is likely to be produced, as observed in patient F2-I-5 in the untreated state.[23] Whether 24-hydroxylated vitamin D metabolites have health consequences, particularly fracture healing in patients with this disease, is still unknown.[24]

With the inclusion of 2 new families, this article extends the number of *CYP2R1* mutations responsible for VDDR1B to 5. We noted the difficulties in establishing the correct diagnosis: First, patients presented with low 25-OH-D levels and were treated as having classical nutritional vitamin D–deficiency rickets; second, the patients had normal/high 1,25-$(OH)_2D$ levels, leading to diagnostic uncertainty. The administration of calcifediol, which bypasses the enzymatic defect, is the adequate treatment to restore optimal vitamin D status.

Disclosures

All authors state that they have no conflicts of interest.

Acknowledgments

We thank the patients and their families, who kindly consented to participate, and Délégation Recherche Clinique et Innovation (DRCI) - CHU de Caen. Grant support is from Délégation Recherche Clinique et Innovation (DRCI) - CHU de Caen APRIM - réf. CHU 16-048. The LC-MS/MS instrument used in these studies was provided by the Waters Corporation (Milford MA) as part of a Queen's University/Waters Corporation Research Agreement.

Authors' roles: AM performed the genetic analyses of *CYP2R1*, generated all figures and tables, and wrote the first draft of the manuscript. AW collected all laboratory data and wrote the first draft of the manuscript together with AM. GA, NC, NR, and HM helped with nucleotide sequence analyses and microsatellite analyses. JCD, AS, PJ, GW, and FF diagnosed and treated patients. BD performed all biochemical assays. LM performed radiological explorations. MK performed LC-MS/MS assays. ND performed functional in vitro analyses. M-LK and FF conceived of the project. FF was the principal investigator. M-LK wrote the final version of the manuscript. GJ reviewed and corrected the final version of the manuscript.

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Etude de *CYP27B1*

L'étude de la cohorte de patients porteurs d'un phénotype HVD a permis l'identification d'une variation c.496G>C / p.Val166Leu (rs8176344) (NM_000785.4) du gène *CYP27B1* à l'état hétérozygote chez un patient de 34 ans exploré en raison d'une hypercalcémie aigue compliquée d'une pancréatite aigüe et d'une néphrocalcinose. Cette variation a précédemment été publiée comme variation gain de fonction[260].

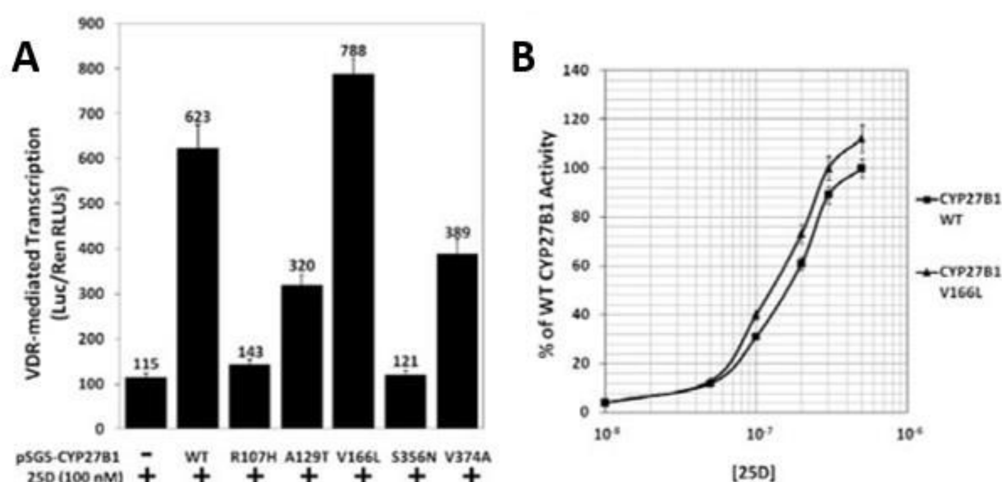

Figure 20 : Etude de fonctionnalité du variant CYP27B1 p.Val166Leu publiée par Jacobs *et al.*

Une variation rare hétérozygote dans le gène *SLC34A3* NM_080877.2:c.1283C>T (p.Ala428Val) (rs775653752) considérée comme de signification indéterminée a également été identifiée chez ce patient. Enfin, un dosage de l'enzyme de conversion de l'angiotensine modérément augmentée pourrait être en faveur d'une hypercalcitriolémie d'origine extrarénale et acquise.

Rapport 25-OH-D / 24,25-$(OH)_2$D dans le VDDR1A

Un échantillon de sérum a pu être collecté pour trois sujets porteurs de mutations bialléliques de *CYP27B1* précédemment identifiées dans le laboratoire (**Tableau 5**).

Sexe	Age (ans)	Traitement	Mutation *CYP27B1*	25-OH-D_3 (nmol/L)	24,25-$(OH)_2D_3$ (nmol/L)	Rapport (N<50)
M	34	aucun	c.1319_1325dupCCCACCC c.196-2A>G (épissage)	169,5	1,7	99,7
F	2	1α-OH-D_3	c.1319_1325dupCCCACCC c.581G>T/p.Gly194Val	163,5	6,7	24,4
F	15	1α-OH-D_3	c.406del/p.Arg136Glyfs*23 c.406del/p.Arg136Glyfs*23	69,4	17,5	3,96

Tableau 5 : Evaluation du rapport 25-OH-D_3 / 24,25-$(OH)_2D_3$ chez des patients VDDR1A

Etude de *CYP24A1*

L'objectif de ce travail était d'identifier de nouvelles variations pathogènes de *CYP24A1*, de préciser le phénotype associé aux variations hétérozygotes de ce gène, une forme autosomique dominante de déficit en 24-hydroxylase ayant été évoquée.

Mutations de *CYP24A1* dans une cohorte de patients hypercalcémiques : des arguments en faveur d'une maladie récessive autosomique

CYP24A1 Mutations in a Cohort of Hypercalcemic Patients: Evidence for a Recessive Trait

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Cet article paru en 2015 dans *Journal of Clinical Endocrinology and Metabolism* porte sur la description d'une cohorte de 72 patients présentant un phénotype clinique et biologique compatible avec une dérégulation du taux de 1,25-$(OH)_2D$ indépendante de la PTH, potentiellement explicable par un défaut de catabolisme de la vitamine D ou une activation anormale de la 1α-hydroxylase. Il s'agit de la description de la plus large cohorte de patients porteurs d'un déficit en vitamine D 24-hydroxylase confirmé par analyse génétique actuellement publiée.

Outre l'identification et la caractérisation de nouvelles variations pathogènes, l'étude des profils biochimiques des patients a permis de mieux caractériser le profil biochimique de cette affection (PTH basse et adaptée, 25-OH-D normale voire haute, 1,25-$(OH)_2D$ inadaptée, 24,25-$(OH)_2D$ basse voire indétectable) et de valider l'intérêt du dosage de la 24,25-$(OH)_2D$ (et en particulier du ratio 25-OH-D / 24,25-$(OH)_2D$, élevé chez les patients atteints) en routine diagnostique.

L'étude de sujets apparentés hétérozygotes est en faveur d'une dérégulation du métabolisme de la vitamine D avec élévation du taux de 1,25-$(OH)_2D$ sans anomalie de la 24,25-$(OH)_2D$ (avec un ratio normal). Le phénotype clinique de ces sujets est variable, la plupart étant asymptomatique.

CYP24A1 Mutations in a Cohort of Hypercalcemic Patients: evidence for a recessive trait

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Context: Loss-of-function mutations of *CYP24A1* (which encodes the 25-OH-D_3-24-hydroxylase) have recently been reported to cause hypercalcemia.

Objectives: The aims of this study were: (a) to evaluate the frequency of *CYP24A1* mutation in patients with medical history of hypercalcemia; (b) to show the clinical utility of a simultaneous assay of serum 25-hydroxyvitamin D_3 (25-OH-D_3) and 24,25-dihydroxyvitamin D_3 (24,25-$(OH)_2D_3$) by liquid chromatography tandem mass spectrometry (LC-MS/MS); (c) to investigate biochemical parameters in heterozygous gene-carriers with *CYP24A1* mutations.

Patients and Methods: We screened for a *CYP24A1* mutation in 72 patients with serum calcium level >2.6 mmol/L and PTH levels <20 pg/mL and recruited 24 relatives after genetic counselling for subsequent investigations. Vitamin D metabolites concentrations were assessed in a subset of patients by LC-MS/MS and results expressed as a ratio (R) of 25-OH-D_3:24,25-$(OH)_2D_3$.

Results: Twenty five patients with hypercalcemia (35%) harbored *CYP24A1* variations. Twenty (28%) had biallelic variations, mostly found in subjects with nephrocalcinosis or renal stones (19/20). Five patients, all neonates, were heterozygous, without renal disease. We described 15 new variations leading to a loss-of-function according to pathogenicity prediction programs, and we characterized functionally 5 of them *in vitro*. A dramatic increase of R, usually >80, was found in patients harboring biallelic mutations providing evidence *in vivo* for the loss of CYP24A1 activity. In contrast, R value remains <25 in patients without *CYP24A1* mutation. Subjects carrying one mutant allele, hypercalcemic ones, as well as gene-carrier relatives, had detectable 24,25-$(OH)_2D_3$ level and a R value <25 indicating normal 24-hydroxylase activity.

Conclusion: *CYP24A1* bi-allelic mutations are frequently found in patients presenting with hypercalcemia, low PTH and renal disease. We confirm the accuracy and effectiveness of a novel blood test estimating the ratio between relevant vitamin D metabolites as a useful screening tool for *CYP24A*1 mutations. Haploinsufficiency is not associated with CYP24A1 deficiency.

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA
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Received December 13, 2014. Accepted July 22, 2015.

Abbreviations:

THE JOURNAL OF CLINICAL ENDOCRINOLOGY & METABOLISM JCEM EARLY RELEASE: ENDOCRINE SOCIETY

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Over the past decade, there has been an increased focus on the benefits of vitamin D for the maintenance of bone health and the possible prevention of chronic disease states such as cancer and cardiovascular disease. To render it active, vitamin D requires two steps of hydroxylation, the first in the liver catalyzed by a 25-hydroxylase (*CYP2R1*, 11p15.2) and the second one in the kidneys, by 1α-hydroxylase (*CYP27B1*, 12q13.1), to produce the hormonal form 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D). This hormonal form plays an important role in calcium and phosphate homeostasis by increasing intestinal calcium absorption and bone resorption and improving the efficiency of renal calcium reabsorption, in addition to playing a role in the regulation of cellular proliferation (1). Catabolism of vitamin D follows two different pathways beginning with 24- or 23-hydroxylation, both including several steps of hydroxylation catalyzed by the mitochondrial enzyme, 25-hydroxyvitamin D-24-hydroxylase (*CYP24A1*, 20q13.2) expressed in most vitamin D target tissues (2).

The variability in serum 25-hydroxyvitamin D (25-OH-D) concentration and the resulting hypercalcemia for a given amount of vitamin D need to be interpreted in light of recent developments in genetics. In 2011, Schlingmann et al (3) identified loss-of-function mutations in *CYP24A1* in a population of children presenting with Idiopathic Infantile Hypercalcemia (IIH) most of them receiving vitamin D supplementation suggesting an unusual sensitivity to vitamin D (4). Common features of this condition included symptomatic hypercalcemia with suppressed parathyroid hormone (PTH) associated to nephrocalcinosis. Since then, additional cases have been published and the phenotypic spectrum has been extended to adults with renal disease (5–16). In addition, while autosomal recessive inheritance was found, other authors have proposed potential dominant inheritance pattern (7).

As only case-reports have been published, we describe here a cohort of patients with a medical history of acute or chronic hypercalcemia, and PTH < 20 pg/mL to better define the phenotype of patients who should benefit of *CYP24A1* genetic screening. In addition, we also show that simultaneous measurement of vitamin D metabolites by liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a valuable screening tool for these patients (17). Lastly, we investigated patients harboring *CYP24A1* mutation in a heterozygous state to better understand the role of *CYP24A1* haploinsufficiency in the occurrence of hypercalcemia.

Patients and Methods

Patients

We enrolled for this study 72 patients (index cases) presenting with hypercalcemia (>2.6 mmol/L), or with a medical history of acute or chronic hypercalcemia, and PTH levels < 20 pg/mL, recruited over an 18 months period in our laboratory. They were considered for the evaluation of *CYP24A1* mutations frequency. After genetic counseling, we also included 24 relatives of the patients, and eleven patients from other centers for genetic screening and clinical and biological investigations. (Table 1 and Supplemental Table 1). For each individual, written informed consent was obtained from the patients and/or their parents for collection of clinical and laboratory data, and for DNA collection to conduct molecular studies.

Biochemical and clinical parameters

Data on clinical symptoms and biochemical parameters (calcium and phosphate in serum and urine, renal function, PTH and vitamin D levels) were collected retrospectively using records from hospitals or primary care physicians. Routine biochemical assays were performed at the time of the diagnosis of acute hypercalcemia, or during follow up after normalization of the calcium level, in a variety of different clinical laboratories. As routine assays for vitamin D cannot discriminate between vitamin D_3 and D_2 we use the term "vitamin D" to describe these measurements. Medullary nephrocalcinosis was assessed by renal ultrasonography (echogenic renal pyramids). Nephrolithiasis was defined as kidney stones on ultrasonography or a medical history of renal colic.

Table 1. Breakdown of the 72 patients presenting with hypercalcemia and their 24 relatives ***** This patient was considered as harboring variations that did not cause IHH and included in group A. Vitamin D metabolites concentrations were assessed in a subset of patients by LC-MS/MS. Patients from other center underwent LC-MSMS analyses by the same laboratory

		Genetic classification			
		A no mutation	**B** biallelic mutation	**C** heterozygous mutation	**D** heterozygous mutation
patients with hypercalcemia and PTH <20pg/mL n = 72	**group 1:** isolated hypercalcemia	11		1	
	group 2: with hypercalciuria	20	1	4	
	group 3: with nephrolithiasis	3	4		
	group 4: with nephrocalcinosis	14	14		
relatives n = 24		1*	2		21
		LC-MS/MS analysis			
our cohort		14	6	3	14
other centers			10	1	

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Calcium absorption studies (Pak test) (18)

Calcium absorption studies were performed as follow: patients remain on a low-calcium diet for 5 days; after an overnight fast, calcium, phosphate and PTH were evaluated in blood and calcium and creatinine in urine before (T0) and 4 hours (T4) after oral calcium administration (one gram). Results are expressed as the difference (T4-T0) of the urine Calcium to Creatinine molar ratio. Results less than 0.6 are considered as normal.

Simultaneous assay of multiple vitamin D metabolites

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) analysis was performed as previously described using 100 μL of serum. Results are expressed as the molar ratio of 25-OH-D_3: 24,25-$(OH)_2D_3$ (R ratio) (17). Values under 25 indicated no defect in 25-OH-D_3-24-hydroxylase activity and were considered as normal.

Molecular analysis

Genomic DNA was isolated from peripheral blood leukocytes using standard methods. We designed several sets of primers for amplification of *CYP24A1* exons 1–11 and their intron-exon junctions as previously described (10). PCR products were purified and sequenced using the CEQ™DTCS Quick Start Kit (Beckman Coulter) on a Beckman Coulter DNA Sequencer. New variations of sequence were searched on *dbSNP* database, and interpreted according to pathogenicity prediction programs (PolyPhen-2, Align-GVGD, MutationTaster, SIFT) to estimate the deleteriousness of each variant. Allelic frequencies were evaluated through the examination of Exome Variant Server database in European American (EA) and African American (AA) population.

Transfection, Cell Culture and analysis of mutant CYP24A1 enzyme activity

Site directed mutagenesis of wild-type *CYP24A1* allowed us to recreate, transfect and test CYP24A1 enzyme activity in V-79–4 Chinese hamster lung fibroblasts using detection of 24- and 23-hydroxylated products of 1,25-$(OH)_2D_3$ by LC-MS/MS as a measure of loss-of-function mutation as previously described (3). (Supplemental Material: appendix 1)

Statistical analysis

Sex was not considered a factor in the statistical analysis of the data. For each biochemical parameter, differences between groups were calculated using the Mann and Whitney nonparametric U test. A two-tailed p value < 0.05 was considered statistically significant. Kruskal-Wallis test was used for multiple group comparison (one way nonparametric ANOVA).

Results

1- Patients

We enrolled 72 patients (38 males (53%) and 34 females (47%)), including 57 children (79%) (range: 1 day – 16 years; mean 2.1 year) and 15 adults (21%) (range: 19–81 years; mean 43.9 years). Four of these patients were previously described (10, 16). Familial occurrence was recorded in 17 patients (24%). Patients were classified into 4 phenotypic groups according to their biological data and the presence of renal disease (Table 1 and Supplemental Table 1). The different cutpoints are as follow: urine calcium:creatinine ratio normal level: adults < 0.55; children under 6 months < 2.4; children 7–12 months $<$ 1.7; children 1–5 years < 1.1 and children above 5 years $<$ 0.7.

Group 1: without hypercalciuria: n = 12 (16%)

Group 2: with hypercalciuria: n = 25 (35%)

Group 3: with nephrolithiasis: n = 7 (10%)

Group 4: with nephrocalcinosis: n = 28 (39%) (including 3 patients with nephrolithiasis and nephrocalcinosis)

Vitamin D metabolites including 25-OH-D_3 and 24,25-$(OH)_2D_3$ were determined by LC-MS/MS in 48 subjects : 23 patients from this cohort, 14 heterozygous relatives and 11 patients recruited from other centers explored with the same method (Table 1)

p.Trp134Gly(19)
p.Glu143del(3,6,9)
p.Leu148Pro(8)
p.Ile149Glyfs*31(3)
p.Glu151X(3)
p. Arg159Gln(3)
p.Trp210Arg(11)
p.Gln315*(19)
p.Glu322Lys(3)
p.Arg396Trp(3)
p.Leu409Ser(3,6,8)
p.Cys477Leufs*14(3)
Deletion (10)
p.Pro21Argfs*8
p.Trp155*
p.Arg157Trp
p.Met253Lys
p.Glu322Ala
p.Thr330Met
p.Leu335Profs*11
p.Met374Thr
p.Cys380Arg
p.Pro389Leu
p.Arg396Gln
p.Val403Phefs*15
p.Arg439Cys
p.Gly456Arg
p.Glu469Alafs*22

Figure 1. ***CYP24A1*** **mutations in patients with hypercalcemia (present study and literature).** Schematic representation of the *CYP24A1* gene: exons are identified as rectangle, coding sequence in white, untranslated sequence in gray. Intronic sequences are represented by straight solid lines between exons. Below, mutations identified in this study; above, mutations reported in the literature. Numbers indicate author references. Recurrent mutations in bold characters; functionally characterized mutations are underlined.

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2- CYP24A1 mutations are found in patients presenting with hypercalcemia

The 72 patients were screened for a *CYP24A1* mutation. They were classified into different groups according to their genotype (Table 1). No mutation was found in 47 patients (group A). We identified 25 patients (35%) harboring variations in the coding sequence of *CYP24A1*. Twenty patients (28%) have biallelic variations (group B) either in a homozygous state (n = 9) with history of consanguinity in 5 cases or in a compound heterozygous state (n = 11). We found only one variation (heterozygous status) in 5 patients (7%) (group C). (Table 2). Further investigations in relatives identified 21 heterozygous (group D). *CYP24A1* variations were identified in most of the 11 exons, with the previously described two hot spots: c.427 429del / p.Glu143del and c.1226T>C / p.Leu409Ser in exon 2 and 9 respectively and functionally characterized by Schlingmann et al (3) (Figure 1).

We described 15 new variations. Five result in truncated protein if translated (frameshift or nonsense): c.62del / p.Pro21Argfs*8; c.464G>A / p.Trp155*; c.1003dup / p.Leu335Profs*11; c.1206del / p.Val403Phefs*15; and c.1406 1407del / p.Glu469Alafs*22. Ten were missense variations c.469C>T / p.Arg157Trp; c.758T>A / p.Met253Lys; c.965A>C / p.Glu322Ala; c.989C>T / p.Thr330Met;

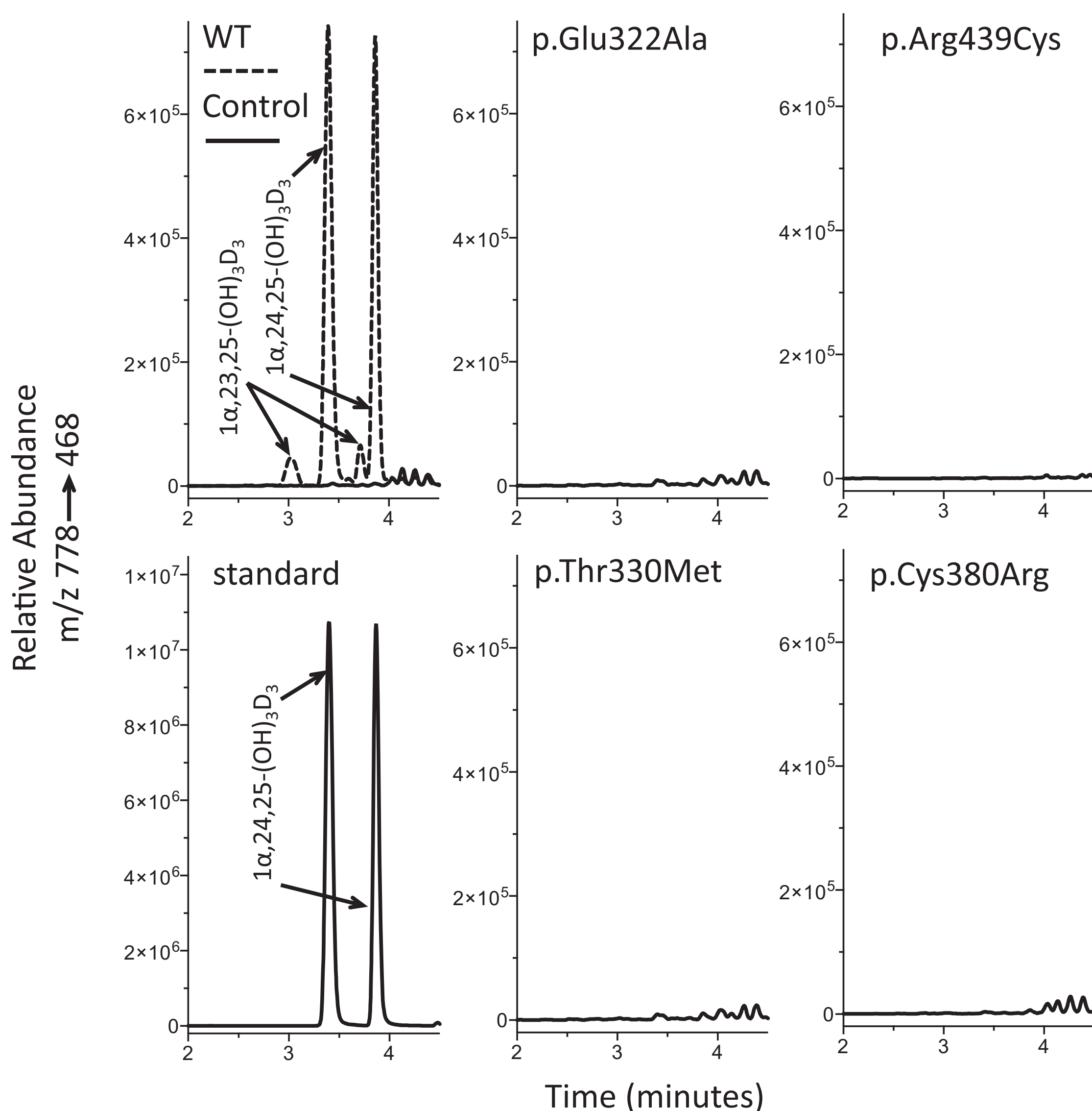

Figure 2. Enzyme activity for mutant CYP24A1 enzyme: LC-MS/MS of DMEQ-TAD-derivatized trihydroxyvitamin D_3 metabolites detected using multiple reaction monitoring (MRM) at m/z 778-> 468. V79–4 Chinese hamster lung fibroblasts were transiently transfected with wild type (WT) human CYP24A1 and mutant CYP24A1 types as indicated and then incubated with $1,25\text{-}(OH)_2D_3$ as described previously (3). Metabolic products of $1,25\text{-}(OH)_2D_3$ were extracted and derivatized using the Cookson reagent, DMEQ-TAD. DMEQ-TAD produces two adducts for each vitamin D metabolite present. Each chromatogram depicts the 2–4.5 minutes portion of the LC showing both adducts of $1,24,25\text{-}(OH)_3D_3$ and $1,23,25\text{-}(OH)_3D_3$. The two adducts of $1,24,25\text{-}(OH)_3D_3$ can be observed at retention times of 3.4 & 3.85 minutes and two adducts from $1,23,25\text{-}(OH)_2D_3$ at retention times of 3.0 & 3.7 minutes.

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Table 2. Pedigree, clinical and biochemical data of heterozygous hypercalcemic patients at the time of diagnosis and during the follow up. M: Male; F: Female; A: African origin; C: Caucasian origin. SGA: Small for Gestational Age. Normal ranges: serum calcium level: before 1 y: [2.1–2.8 mmol/liter], after 1 y: [2.2–2.6 mmol/liter]; PTH between 2 and 4 y: [5.7–34.2 pg/mL (male) and 3.6–32 pg/mL (female)]; 25-OH-D: [50–200 nmol/liter]; 1,25-$(OH)_2$-D: [50–160 pmol/liter]; ratio: [5–25] (17). d: day(s); mth: month(s); y: year(s)

pedigree	Sex/origin	Age at diagnosis	Circumstances of diagnosis	Maternal Vitamin D supplementation	Daily Vitamin D supplementation	Genotype	Follow up		
not available	M/A	1 d	apnea Ca = 2.84 1,25-$(OH)_2$D = 118 at d18	no	yes	c.1121T>C (p.Met374Thr)	3.5 mth: Ca = 2.67 *P* = 2.1 PTH = 17 25-OH-D = 97.3 1,25-$(OH)_2$D = 237.6	21 mth: Ca = 2.61 *P* = 1.66 PTH = 31 25-OH-D = 64.9 1,25-$(OH)_2$D = 148.8	2 y 9 mth: Ca = 2.61 *P* = 1.75 PTH = 27 25-OH-D = 59.9 1,25-$(OH)_2$D = 144
	M/A	3 d	infection Ca = 2.71; 3.11 at d8 *P* = 1.59 PTH = 5 25-OH-D = 46.2	not available	yes	c.1121T>C (p.Met374Thr)	3 mth: Ca = 2.7 25-OH-D_3 = 76.4	8mth: 25-OH-D_3 = 31.8 24,25-$(OH)_2D_3$ = 1.5 Ratio = 21.2	
	M/C	11 d	preeclampsia prematurity (32w) Ca = 2.93 PTH = 1.6 25-OH-D = 75.6 1,25-$(OH)_2$D = 422.4	yes, maternal hypercalcemia and suppressed PTH	no	c.965A>C (p.Glu322Ala)	4 mth: Ca = 2.77 *P* = 2.01 25-OH-D_3 = 38.5 24,25-$(OH)_2D_3$ = 2.7 Ratio = 14.3	2 y: Ca = 2.64 *P* = 1.52 PTH = 25 25-OH-D = 107.3 1,25-$(OH)_2$D = 93.6	
	F/C	4 d	Prematurity (34 w) Ca = 3.16 PTH = 3.1 25-OH-D = 132.3 1,25-$(OH)_2$D = 386.4	not available	yes	c.1315C>T (p.Arg439Cys)	16 mth: 25-OH-D_3 = 9.1 24,25-$(OH)_2D_3$ = 0.5 Ratio = 18.2		
not available	F/A	13 d	SGA Ca = 2.83 PTH = 1.4	yes	yes	c.1121T>C (p.Met374Thr)	not available		

c.1121T>C / p.Met374Thr; c.1138T>C / p.Cys380Arg; c.1166C>T / p.Pro389Leu; c.1187G>A / p.Arg396Gln; c.1315C>T / p.Arg439Cys and c.1366G>C / p.Gly456Arg. The frequency and the deleteriousness of each variant is represented in Supplemental Table 2. Glu322 and Arg396 were described with other substitutions (c.964G>A / p.Glu322Lys and c.1186C>T / p.Arg396Trp) and loss of enzymatic activity (3). While there was easily detectable production of 1,24,25-$(OH)_3D_3$ and 1,23,25-$(OH)_3D_3$ with the wild-type enzyme, the following mutants, p.Thr330Met, p.Cys380Arg and p.Arg439Cys were totally devoid of enzyme activity. The p.Met374Thr variant was found in 4 patients of African origin. Its high estimated frequency (5.2% heterozygosity) in this population and the functional "in vitro enzyme assay" were suggestive of a polymorphism rather than a loss-of-function mutation. We found this p.Met374Thr variant associated to another variant (p.Arg157Trp), also described as a polymorphism (1.9% heterozygosity), in a girl presenting with typical IIH symptoms and nephrocalcinosis (patient n°7, Supplemental Table 1). The same genetic combination was found in her asymptomatic two months old brother who had normal serum calcium and PTH levels, normal urine calcium level, and no renal disease. Evaluation of serum vitamin D metabolites in this p.Met374Thr/p.Arg157Trp subject confirmed a normal R ratio suggesting the presence of one of these being a polymorphism rather than a mutation. Furthermore, though p.Arg157Trp remains untested in the in vitro CYP24A1 assay, both p.Met374Thr and p.Arg157Gln variants show measurable enzyme activity in vitro suggesting that both amino acid residues are tolerant of variation (2). Thus, this patient was considered as harboring variations that did not cause IHH and included in group A.

3- Clinical data of patients with biallelic *CYP24A1* mutations

Biallelic mutations were mostly found in the groups of patients with renal disease (18/35 (51%) either renal stones (4/7 = 57%, group 3) or nephrocalcinosis (14/28 = 50%, group 4). Children with *CYP24A1* biallelic mutations presented with early (mean age at diagnosis: 8.5 months, median age at diagnosis: 5.5 months), severe (3.33 mmol/L [2.47–4.94]) and symptomatic (mainly weight loss and feeding problems) hypercalcemia. All children exhibited a renal disease (groups 3 and 4), mainly nephrocalcinosis (8/9).

4- Biochemical parameters in patients without mutation compared to patients with biallelic mutations (Figure 3, groups A and B)

In order to improve genetic screening, we searched for specific clinical and/or biological data between patients without mutation (group A) and patients with *CYP24A1* biallelic mutation (group B). To improve our statistical analysis, we included 10 other patients with biallelic mutation recruited from other laboratories who underwent LC-MS/MS analysis by the same laboratory (Supplemental Table 1). Serum calcium and PTH levels in patients from group A and B are similar, but routine assay of 1,25-$(OH)_2$D concentration is slightly higher in patients without mutation than those with biallelic mutations (P = .0322). The mean 25-OH D_3 level in group A is below the

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normal range (42.5 nmol/L). In contrast, in group B, the 25-OH D_3 level is higher and the 24,25-$(OH)_2D_3$ level is lower than in group A ($P < .0001$ and $P = .0006$, respectively). Last, while R is normal in group A, the patients of group B exhibit a dramatic increase in R providing evidence "in vivo" for the loss of CYP24A1 enzyme activity.

5- Clinical and Biochemical parameters in patients with *CYP24A1* mutation in a heterozygous state

5–1 CYP24A1 variations were found in a heterozygous state in five patients with hypercalcemia (Table 2 and Figure 3 group C).

These patients were neonates, under 2 weeks (range 1 to 13 days). Hypercalcemia was found during routine exams performed for another pathology: prematurity, growth retardation, infection or apnea. None presented with renal pathology. One child was born prematurely to a mother presenting with severe hypercalcemia and suppressed PTH level during the pregnancy and subsequent investigations found biallelic *CYP24A1* mutation in his mother. We described two new mutations c.965A>C / p.Glu322Ala and c.1315C>T / p.Arg439Cys, both tested as a loss-of-function mutation (Figure 2). However, we found the same variation c.1121T>C / p.Met374Thr in 3 patients all from African origin and described as a polymorphism in this population. The presence of heterozygous polymorphisms in several exons ruled out a heterozygous deletion that was not detected by sequence analysis. Calcium, PTH and 1,25-$(OH)_2D$ levels were similar to patients from group A and B. They have normal 25-OH-D_3 level, measurable amounts of 24,25-$(OH)_2D_3$ and normal R ratio suggesting functional CYP24A1 activity.

5–2 Clinical and Biochemical parameters in relatives with CYP24A1 heterozygous mutation (Figure 3 group D)

In order to better understand the role of *CYP24A1* mutation at the heterozygous state in the occurrence of hypercalcemia, we investigated 21 adult relatives (9 females and 12 males, mean age 36.5 years) recruited after genetic counseling of affected patients and harboring one CYP24A1 mutation (Supplemental material 1). They have normal serum calcium and PTH levels. The 1,25-$(OH)_2D$ level is not significantly different from that of heterozygous patients (group C). Again, simultaneous assay of both 25-OH-D_3 and 24,25-$(OH)_2D_3$ provides evidence for the presence of normal CYP24A1 activity with a R ratio within the normal range, similar to patients of group C.

One family was of particular interest (Figure 4 and Table 3). In this family, the proband (II-1) was followed for renal failure and referred to our laboratory for evaluation of intermittent hypercalcemia (up to 4.4 mmol/L) with suppressed PTH in a context of vitamin D supplementation. Neither nephrocalcinosis nor nephrolithiasis was noticed on ultrasonography. He harbors two *CYP24A1* mutations (c.443T>C / p.Leu148Pro and c.1187G>A / p.Arg396Gln) that were also found in his brother (II-3). One mutation was transmitted to his seven asymptomatic children who had calcium, ionized calcium, PTH and 25-

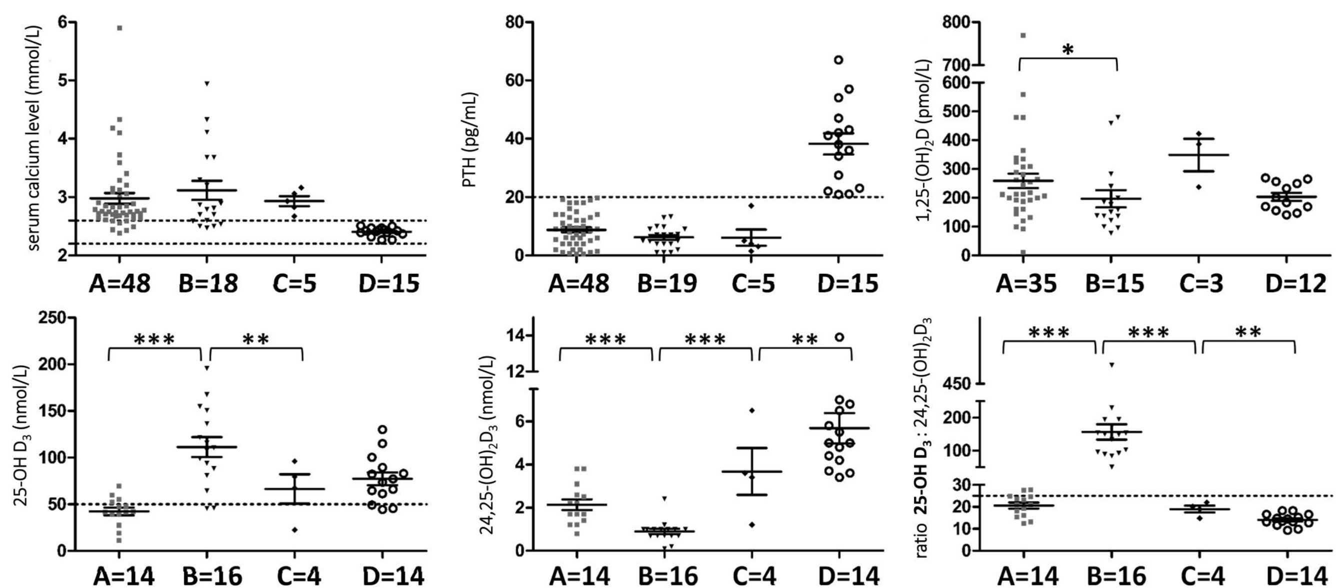

Figure 3. Biochemical data and LC-MS/MS analysis of serum vitamin D metabolites and ratio according to the different genotypes. Group A: hypercalcemic patients without *CYP24A1* mutation; group B: hypercalcemic patients with biallelic *CYP24A1* mutation; group C: hypercalcemic patients with heterozygous *CYP24A1* mutation; group D: relatives with *CYP24A1* mutation in a heterozygous state. * $P \le .05$; ** $P \le .01$ ***; $P \le .001$. The dash horizontal lines indicate the reference limit. Normal individuals with a 25-OH-D_3 of 50–125 nmol/L exhibit a range for 24,25-$(OH)_2D_3$ of 3.6–12 nmol/L and the normal ratio (R) of 25-OH-D_3:24,25-$(OH)_2D_3$ is between 5–25 (17).

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Table 3. Clinical and Biochemical data at the time of diagnosis, during the Pak test and simultaneous LC-MS/MS analyses of serum vitamin D metabolite and ratio according to the genotype G2: group 2; AS: asymptomatic; -: no data; NL: nephrolithiasis (renal stones: x episodes); PA: parathyroid adenoma. # Patient presenting with PA. * Normal ranges are indicated according to Kaufmann *et al.* 2014 (17). N: normal *CYP24A1* sequence; M: *CYP24A1* mutation; M/N: Heterozygosity

patient		**II-1**	**II-2**	**II-3**	**II-7**	**II-8**	**III-1**	**III-2**	**III-3**	**III-4**	**III-5**	**III-6**	**III-7**	**III-8**	**IV-1**	**IV-2**	**IV-3**	**IV-4**
genotype		**M/M**	**N/N**	**M/M**	**M/N**	**M/N**	**M/N**	**M/N**	**M/N**	**M/N**	**M/N**	**M/N**	**M/N**	**M/N**	**M/N**	**M/N**	**M/M**	**M/N**
age (years)		**81**	**73**	**70**	**85**	**73**	**51**	**50**	**48**	**46**	**44**	**36**	**47**	**50**	**22**	**19**	**18**	**10**
sex (M/F)		M	F	M	M	M	M	M	F	F	F	F	F	M	M	M	M	F
symptoms		G2	NL (1)	NL	AS	PA	AS	AS	AS	AS	AS	AS	NL (1)	AS	AS	AS	NL (2)	AS
calcium 2.2–2.60 mmol/liter		2.59	2.41	2.43	2.42	2.79	2.41	2.47	2.37	2.44	2.32	2.42	2.27	2.27	2.39	2.5	2.52	2.51
PTH 12–88 pg/mL		**9.8**	37	**14**	57	102	54	23	67	36	47	38	21	41	34	22	**7**	42
25-OH-D 50–125 nmol/liter		84.4	56.7	187.2	98.1	65.6	105.8	42.9	55.9	40.9	56.7	41.4	97.3	32.4	27	44.4	45.4	50.9
1,25 $(OH)_2$-D 48–160 pmol/liter		238.2	142.8	-	215	378.7	236.4	130.2	135.6	240	169.2	186.7	156	144	156.7	230.6	155.5	248.6
PAK test																		
calcium 2.2–2.60 mmol/liter	T_0	-	-	-	2.31	-	2.31	2.32	2.03	1.91	2.24	2.29	2.26	2.06	-	-	-	-
	T_{60}	-	-	-	2.39	-	2.33	2.40	2.38	2.11	2.37	2.48	2.40	2.22	-	-	-	-
ionised calcium 1.2–1.3 mmol/liter	T_0	-	-	-	1.21	-	1.21	1.05	1.13	1.17	1.16	1.13	1.17	1.10	-	-	-	-
	T_{60}	-	-	-	1.24	-	1.15	1.21	1.16	1.21	1.21	1.16	1.20	1.11	-	-	-	-
PTH 12–88 pg/mL	T_0	-	-	-	58	-	62	36	31	29	39	26	26	30	-	-	-	-
	T_{60}	-	-	-	41	-	35	16	14	12	14	9	10	25	-	-	-	-
Urine calcium: creatinine (Δ <0.6)	T_0	-	-	-	0.37	-	0.14	0.14	0.47	0.35	-	0.46	0.39	0.08	-	-	-	-
	T_{240}	-	-	-	0.43	-	0.26	0.65	0.96	0.69	0.59	1.01	1.47	0.56	-	-	-	-
	Δ	-	-	-	0.06	-	0.12	0.51	0.49	0.34	-	0.55	1.08*	0.48	-	-	-	-
Simultaneous assay of serum Vitamin D metabolites																		
25-OH-D_3 >50 nmol/liter		-	-	-	44.4	-	82.4	61.2	89.6	73.6	100.3	83.1	130.0	42.4	-	-	-	-
24,25$(OH)_2D_3$ nmol/liter		-	-	-	3.4	-	6.5	4.3	6.7	4.8	5.5	5.0	13.9	3.6	-	-	-	-
Ratio (<25)		-	-	-	13.2	-	12.7	14.2	13.3	15.3	18.2	16.5	9.3	11.8	-	-	-	-

OH-D levels within the normal range. However, it is worthy to note that in this family, 8 of 13 heterozygous subjects show slightly elevated 1,25-$(OH)_2$D levels. Genetic counseling revealed on the one hand a cousin (II-8) presenting with parathyroid adenoma and on the other hand his wife (II-2), one of his daughters (III-7) and one of his grandsons (IV-3) complaining of renal stones. Surprisingly, genetic analysis of IV-3 found two mutations: one inherited from his grandfather through his mother (c.443T>C / p.Leu148Pro) and a new mutation c.1186C>T / p.Arg396Trp inherited from his healthy father (III-8). In order to know whether heterozygous *CYP24A1* mutation could be associated with chronic hypercalciuria and/or increased calcium absorption from the intestine due to abnormal 1,25-$(OH)_2$D level, we performed in this family calcium absorption studies (Table 3). The rise in urinary calcium to creatinine ratio was high in the subject III-7 with renal stones, but normal in all the others heterozygous subjects.

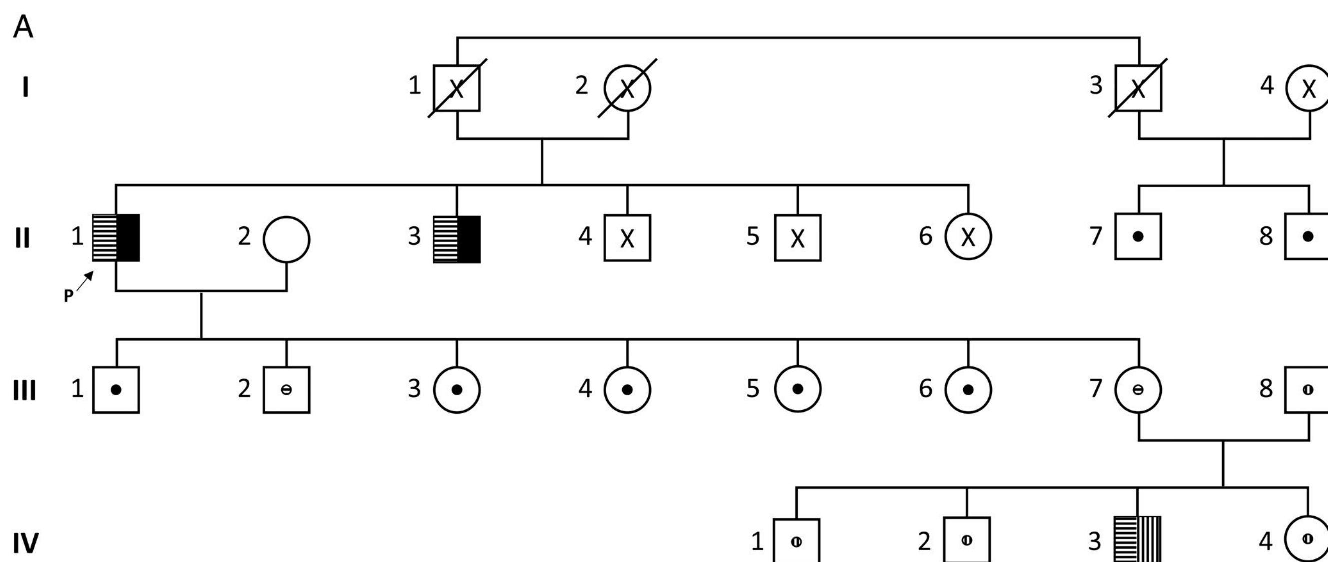

Figure 4. Pedigree of an informative family X: lack of molecular analysis; : compound heterozygous state; : heterozygous carrier Mutations: : c.443T>C; ■: c.1187G>A; : c.1186C>T

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Discussion

This report presents extensive clinical and biochemical data on a large cohort of patients with a newly identified hypercalcemic syndrome characterized by vitamin D hypersensitivity caused by loss-of-function mutations in the *CYP24A1* gene. Our study emphasizes that a loss-of-function *CYP24A1* mutation is frequently associated with serious renal complications including nephrolithiasis and nephrocalcinosis; it demonstrates the accuracy and effectiveness of a novel blood test for the relevant vitamin D metabolites 25-OH-D_3 and 24,25-$(OH)_2D_3$. In addition, it should be noted that in the era of widespread vitamin D supplementation, women with *CYP24A1* defect could develop severe hypercalcemia during pregnancy associated with prematurity and intra-uterine growth retardation (19).

CYP24A1 loss-of-function mutations represent the most recent elucidated cause of hypercalcemia after PTHrP -mediated hypercalcemia of malignancy, hyperparathyroidism, milk-alkali syndrome and vitamin D-mediated hypercalcemia due to either to vitamin D intoxication or to poorly regulated 1α-hydroxylation, as observed in some granulomatous diseases or lymphoma. The field is still developing the most efficient ways to distinguish the various causes of hypercalcemia and our paper points to symptoms to look out for and biochemical techniques that can be used to diagnose and monitor the condition.

Since the first publication (3) reporting the association between *CYP24A1* loss-of-function mutations and IIH, there have been about ten follow-up papers over the past 3 years from several groups in Europe and North America (5–16), corroborating their findings and extending the age of patients into adulthood. In most cases these are reports limited to a single patient up to 6 patients. Consequently, our current report is one of the first on a large cohort in which patients with a medical history of hypercalcemia and low PTH levels were screened for *CYP24A1* mutations. We included hypercalcemic patients with suppressed PTH but normocalcemic patients with inappropriately low PTH ($<$ 20 pg/mL) should be considered, as these two biological pictures could reflect two states of the same disease depending of vitamin D supplementation. Indeed, genetic counseling allowed us to find a patient with normal serum calcium level but low PTH (IV-3, Table 3), harboring however biallelic CYP24A1 mutation, situation already reported (10). Indeed, reduced rates of inactivation of 1,25-$(OH)_2D$ caused by a *CYP24A1* defect result in decreased 1,25-$(OH)_2D_3$ clearance and thus in increased calcium absorption from the intestine, favoring hypercalcemia, which in turn down-regulates PTH secretion through the vitamin D receptor and calcium sensing receptor in the parathyroid cell. Tight balance between calcium load and renal function is also a determinant of plasma calcium levels as absorbed calcium is promptly excreted. Lastly, hypercalcemia and low PTH levels favor calcium excretion by the kidney, favoring nephrocalcinosis, further exacerbated by the hyperphosphaturia caused by high FGF23 levels in response to elevated calcitriol (11, 14).

In this population presenting with IIH we found biallelic mutations with a higher frequency than that described by Dauber et al (6) (19/72 and 1/27 respectively) suggesting that *CYP24A1* mutations are a more frequent cause of idiopathic hypercalcemia in a French population than in a North American one. We confirm the presence of two hot spots found in 50% of cases: c.427 429del / p.Glu143del and c.1226T>C / p.Leu409Ser in exon 2 and 9 respectively and functionally characterized by Schlingmann et al (3).

Two variants, p.Met374Thr and p.Arg157Trp, were considered as polymorphisms rather than mutations, despite *in silico* predicted pathogenicity. On the one hand, they are found in general population with a high frequency (5.2% and 1.9% respectively). On the other hand, p.Met374Thr retained in vitro measurable enzyme activity. At last, evaluation of serum vitamin D metabolites in an asymptomatic subject harboring the p.Met374Thr / p.Arg157Trp combination showed a normal 25-OH-D_3: 24,25-$(OH)_2D_3$ ratio suggesting that at least one of these variants is a polymorphism. Furthermore, a recently identified individual with the p.Glu143del / p.Arg157Trp combination also has a normal R ratio despite the fact that p.Glu143del is a well-known and common mutation (not included in this study). This also suggests that p.Arg157Trp is a polymorphism. Currently we lack a serum sample from an individual with p.Met374Thr coupled with a known deleterious mutation to confirm whether this variant causes IIH.

Renal disease associated with *CYP24A1* starts during infancy. The vast majority of children with biallelic mutations of *CYP24A1* present with nephrocalcinosis as a manifestation of their increased sensitivity to vitamin D, even though they have small dietary vitamin D intakes. Castanet et al (10) suggested that restriction of vitamin D and sun protection might protect affected patients, particularly in the infant period, preventing acute hypercalcemia, as in one family the child who did not receive a vitamin D supplement did not present with hypercalcemia or hypercalciuria, despite having the same homozygous mutation as his older symptomatic brother. This observation conversely highlights the potentially deleterious contribution of vitamin D supplementation in these children. Recently the phenotype has been extended to adults

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with chronic renal disease (13, 15, 16). The lifetime prevalence of nephrolithiasis is high, estimated at 10%–15%, as illustrated in the family described in figure 4 and Table 3, in which subject II-2, without *CYP24A1* mutation, presented with renal stones. Nesterova et al (8) estimated the frequency of kidney stones due to *CYP24A1* deficiency to be between 4%–20%. Given the high number of patients that should be screened for *CYP24A1* mutation, the LC-MS/MS assay for simultaneous measurement of several vitamin D metabolites including 24,25-$(OH)_2D_3$ in serum should constitute a useful screening tool. The ratio of 25-OH-D_3:24,25-$(OH)_2D_3$ allows us to distinguish CYP24A1 defects from hypervitaminosis D during vitamin D intoxication, and was proposed as a more accurate parameter to express the absence of CYP24A1 activity than serum 24,25-$(OH)_2D_3$ alone, especially as some of these patients have low vitamin D status and consequently low 24,25-OH_2D levels (17).

In 17 patients with biallelic *CYP24A1* mutations investigated with the same procedure (including our 15 patients and 2 patients published by Kaufmann et al (17)), serum 24,25-$(OH)_2D_3$ is very low or undetectable and outside of the normal range. However, this parameter alone is unable to distinguish patients with or without *CYP24A1* mutation: it can be misleading if there is vitamin D deficiency with low serum 25-OH-D_3. In our hands, the ratio 25-OH-D_3: 24,25-$(OH)_2D_3$ takes into consideration the circulating 25-OH-D_3 level and provides a clear distinction from vitamin D deficiency. Thus, we suggest that a 25-OH-D_3: 24,25-$(OH)_2D_3$ ratio > 50, and usually > 80, is indicative of IHH due to an inactivating *CYP24A1* mutation in vivo. But the corollary is also true that all patients with a single or wild-type allele have a normal 25-OH-D_3: 24,25-$(OH)_2D_3$ ratio.

The etiology of hypercalcemia in patients with normal 24-hydroxylase activity remains unclear. Normal 25-OH-D_3 levels in these patients do not support vitamin D intoxication, while high 1,25-$(OH)_2D$ levels suggest another metabolic defect (22). We described patients harboring *CYP24A1* mutations in a heterozygous state suggesting the hypothesis of an autosomal dominant trait (7). These findings prompted us to investigate other subjects with one mutation and the most convenient was to study the relatives. In our study, all the heterozygous subjects, gene-carriers and hypercalcemic patients had normal 24-hydroxylase activity as confirmed in vivo by detectable 24,25-$(OH)_2$-D_3 level and a normal R ratio as also found by Meusburger (11). The Pak test also found normal calcium absorption in adult heterozygotes. These investigations suggest their hypercalcemia not due to *CYP24A1* loss of function mutations.

However, heterozygous hypercalcemic neonates, have higher 1,25-$(OH)_2D$ and lower PTH levels than asymptomatic adults suggesting excessive vitamin D intake. We should hypothesize that the consequences of haploinsufficiency could vary during the growth from childhood to adulthood. Indeed, there are a number of functional changes that occur in the kidney that accompany an increase in the glomerular filtration rate (GFR), as the infant matures (20). The initial prematurity in renal function observed during the early infancy could explain a relative sensitivity of heterozygous children to hypercalcemia. Supporting this hypothesis, hypercalcemia in an infant with *CYP24A1* biallelic mutations is most severe in the first days/mo of life when renal function relative to body mass is low (3). In the case of heterozygous mutation, the vitamin D supplementation could exceed the capacity of haploinsufficient 25-OH-D_3-24-hydroxylase activity in children (21). On the other hand, the normal calcium intestinal absorption observed in the adult relatives ruled out the hypothesis of an autosomal dominant inheritance. However, we cannot exclude the role of another currently unidentified factor in the etiology of IIH.

Conclusion

Our findings emphasize the importance of recognition, genetic diagnosis and proper treatment of CYP24A1 defects. Mutations are frequently found in patients presenting with hypercalcemia with low or suppressed PTH especially in patients with nephrocalcinosis or renal stones; inappropriately low PTH level with normal serum calcium level should be also taken into consideration. We confirm the accuracy and effectiveness of a novel blood test estimating the ratio between relevant vitamin D metabolites 25-OH-D_3 and 24,25-$(OH)_2D_3$, which constitutes a useful screening tool for *CYP24A1* mutations. Most of patients with *CYP24A1* heterozygous mutation remain asymptomatic. However, the observation of hypercalcemic patients with such genotype could suggest that hypercalcemia may occur in the setting of excessive vitamin D intake. This study leads to recommend preventive measures including restriction of vitamin D supplementation and dietary intakes that could protect affected relatives particularly during the neonatal period.

Acknowledgments

We would like to thank all the physicians and the patients for their precious collaboration: Dr Quintard-Lacour and Pr Tessier (Nephrology, University Hospital, Limoges); Dr Maisin and Dr Kuzbari (Pediatrics, Robert Debré Hospital, Paris); Pr Linglart and Dr Rothenbuhler (Pediatrics, Kremlin-Bicêtre Hospital, Par-

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is); Dr Boutignon (Compiegne Hospital), Dr Thibault (Pediatrics, University Hospital, Montpellier); Dr Taque (Pediatrics, University Hospital, Rennes); Dr D'Anella (Endocrinology, Avignon Hospital); Dr Dupre (Pediatrics, Valenciennes Hospital); Dr Djaghri (Neonatology, Sainte Camille Hospital, Bry-sur-Marne); Dr Ranchin (Pediatrics, University Hospital, Lyon), Pr Prie (Department of Physiology, Necker University Hospital, Paris).

Glenville Jones is supported by the European Rare Diseases Consortium in conjunction with the Canadian Institutes for Health Research (Grant # ERA-132 931). Glenville Jones also acknowledges the Waters Corporation for the provision of the Xevo TQ-S LC-MS/MS instrument used in these studies.

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Disclosure Summary: The authors have nothing to disclose.
*G Jones and ML Kottler contributed equally to this work
This work was supported by .

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Caractérisation sur le plan moléculaire d'une délétion récurrente de *CYP24A1* de 10,9 kb dans l'Hypercalcémie Infantile Idiopathique

Molecular characterization of a recurrent 10.9 kb CYP24A1 deletion in Idiopathic Infantile Hypercalcemia

L'étude de deux familles particulières nous a permis d'identifier et de caractériser une grande délétion de *CYP24A1* potentiellement non détectée par les techniques de séquençage Sanger usuellement employée. La première famille avait été préalablement publiée par l'équipe de notre laboratoire[251]. Dans ce nouveau travail, publié dans l'*European Journal of Medical Genetics*, nous avons précisé les points de cassure impliqués dans cette délétion récurrente référencée dans les bases de données de CNV, et validé la possibilité de détection de ce remaniement en MPS par un traitement approprié des données de séquençage. Cette délétion a été identifiée dans trois familles supplémentaires (points de cassure et haplotypes identiques) depuis la publication de ce travail.

La présence de remaniements de ce type, potentiellement non détecté par les techniques de séquençage type Sanger, pourrait en partie expliquer les cas de déficit en vitamine D 24-hydroxylase considérés d'hérédité autosomique dominante.

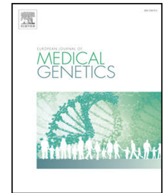
ARTICLE IN PRESS

European Journal of Medical Genetics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

European Journal of Medical Genetics

journal homepage: www.elsevier.com/locate/ejmg



Molecular characterization of a recurrent 10.9 kb *CYP24A1* deletion in Idiopathic Infantile Hypercalcemia

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ARTICLE INFO

Keywords:
Nephrocalcinosis
Vitamin D
Hypercalcemia

ABSTRACT

Loss-of-function mutations in *CYP24A1* (MIM 126065 20q13.2), the gene encoding the 24-hydroxylase responsible for 25-OH-D and 1,25-$(OH)_2$D degradation, are identified in about 20% of patients presenting Idiopathic Infantile Hypercalcemia (IIH) (MIM 143880). Common features of this autosomal recessive condition included hypercalcemia with hypercalciuria, suppressed PTH and a high 25-OH-D_3:24,25-$(OH)_2D_3$ ratio. Medical care mainly relies on sun protection and life-long contraindication of vitamin D to avoid complications such as early nephrocalcinosis and renal failure.

Molecular diagnosis therefore keeps a crucial place in the diagnosis of IIH, and genetic counseling should be systematically recommended to prevent vitamin D administration in affected siblings.

In this report is described the molecular characterization of a *CYP24A1* deletion identified in two unrelated families. This highlights the potential role of *CYP24A1* copy number variations (CNV) in IIH. Considering the presence of CNV affecting *CYP24A1* in public databases, CNV analysis should be systematically added to the sequencing studies in IIH. Targeted Massively Parallel Sequencing (MPS) study coupled with a CNV detection tool called CovCop is a powerful method to detect genic rearrangement and improve genetic analysis.

1. Introduction

Loss-of-function mutations in *CYP24A1* (MIM 126065 20q13.2) are identified in about 20% of patients presenting Idiopathic Infantile Hypercalcemia (IIH) (MIM 143880) (Molin et al., 2015). This gene encodes the mitochondrial enzyme 25-hydroxyvitamin D 24-hydroxylase which catabolizes the vitamin D metabolites. High levels of active vitamin D cause increased calcium intestinal absorption, leading to high serum and urine calcium with suppressed parathyroid hormone (PTH). A high 25-OH-D3:24,25-$(OH)_2$D3 ratio (substrate: product ratio), which directly reflects the enzymatic defect, has been specifically associated with this condition and proposed as a screening tool (ref). In children, common features of this condition include acute symptomatic hypercalcemia, renal stones and nephrocalcinosis (Schlingmann et al., 2011). Medical care mainly includes sun protection and life-long contraindication of vitamin D from childhood, to avoid acute complications of hypercalcemia and chronic hypercalciuria and subsequent renal failure.

Molecular diagnosis therefore has a crucial place in the diagnosis of IIH, and genetic counseling should be systematically recommended to

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https://doi.org/10.1016/j.ejmg.2018.11.011
Received 9 April 2018; Received in revised form 1 October 2018; Accepted 9 November 2018
1769-7212/ © 2018 Published by Elsevier Masson SAS.

Please cite this article as: Molin, A., European Journal of Medical Genetics, https://doi.org/10.1016/j.ejmg.2018.11.011

prevent vitamin D administration in affected siblings. Since the first publication (Schlingmann et al., 2011), the phenotypic spectrum has been extended to adults with renal disease (Castanet et al., 2013; Colussi et al., 2014; Dauber et al., 2012; Dinour et al., 2013; Fencl et al., 2013; Figueres et al., 2015; Jacobs et al., 2014; Meusburger et al., 2013; Molin et al., 2015; Nesterova et al., 2013; Tebben et al., 2012). The vast majority of observations suggested a recessive autosomal inheritance, but some authors have suggested that there might also be a dominant inheritance pattern (Tebben et al., 2012).

Most *CYP24A1* mutations previously reported are point variations, essentially missense, with at least 2 recurrent mutations (Molin et al., 2015). They were detected using targeted sequencing or whole exome sequencing (WES) (Dauber et al., 2012).

This report highlights the requirement of Copy Number Variations (CNV) detection procedures during *CYP24A1* analysis for molecular diagnosis of IIH to search for CNV which could go undetected with conventional Sanger sequencing.

1.1. Patients (Fig. 1)

Family 1 has previously been reported by Castanet et al. (Castanet et al., 2013). The proband (F1-II1) is the first child of a non-consanguineous healthy couple, who received vitamin D (1900 UI/day) until the diagnosis of severe hypercalcemia with hypercalciuria at the age of 6 months. PTH level was low and nephrocalcinosis was detected on ultrasonography. Molecular diagnosis allowed precocious identification of an affected sibling (F1-II3) and prevent vitamin D administration and complications of the disease.

The second proband (F2-II3) (patient 17 in (Molin et al., 2015)) is a 10-year-old girl, the third child of a healthy nonconsanguineous couple. At the age of 14 months, receiving 1200 UI/day of vitamin D, she presented polydipsia and vomiting in a context of postnatal growth failure, hypercalcemia and hypercalciuria with suppressed PTH. Renal ultrasound showed medullary nephrocalcinosis grade 2 (mild increase of echogenicity at whole pyramid). Vitamin D supplementation was ceased.

Biochemical features are included in Supplemental Material Table 1.

Written consent was obtained from the patients and/or their parents for the collection of clinical and laboratory data and for genetic investigation. The molecular analysis of relatives was suggested through genetic counseling after the identification of a mutation in the probands.

2. Methods

Genomic DNA was extracted from whole blood samples with routine procedures.

Two sequencing methodologies were used, i. e. Sanger and targeted Massively Parallel Sequencing (MPS) of genes involved in vitamin D metabolism including *CYP24A1*. Reference sequence (ENST00000216862/NM_000782.4) was obtained from ensembl database (http://www.ensembl.org).

Sanger: The 11 coding exons of *CYP24A1* and their flanking regions were amplified by PCR using specific primers as previously described (Castanet et al., 2013; Molin et al., 2015).

MPS: The exons 1 to 12 were sequenced using a custom Ampliseq library and the Ion Torrent Personal Genome Machine (PGM) (ThermoFisher Scientific, Waltham, Massachusetts, USA). Variants were annotated using IonSuit (ThermoFisher Scientific, Waltham, Massachusetts, USA) and Nextgene (SoftGenetics, State College, Pennsylvania, USA). A read depth (RD)-based approach was used to detect CNV using the CovCop (v1.2 - https://omictools.com/cov-cop-tool) tool (Derouault et al., 2017). This procedure also identified single nucleotide polymorphisms (SNP) which were used to determine pathogenic haplotype at *CYP24A1* locus.

Quantitative PCR: Copy number of exons 9 and 11 was assessed using specific TaqMan probes (Hs00815832_cn targeting the junction between intron 8 and exon 9 and Hs01863481 targeting the junction between intron 10 and exon 11) (ThermoFisher Scientific, Waltham, Massachusetts, USA).

Breakpoints determination: Sixteen primers were designed: a forward primer in exon 9, and 15 reverse primers (R1 to R15) from intron 10 each placed about 800 pb away from other (Supplemental material 1). Amplification was performed following standard routine protocol and PCR products were sequenced.

3. Results

In the proband F1-II1 (family 1) (Castanet et al., 2013), the exon 9 to 11 were not amplifiable. Thus, a deletion which included at least these exons in a homozygous state was suspected. This was coherent with quantitative PCR analysis; the deletion was inherited from both heterozygous parents.

In Family 2, sequencing analysis in the proband F2-II3 found the recurrent exon 9 mutation p.Leu409Ser in an apparently homozygous state. However parental analyses revealed that it was in a heterozygous state in the maternal DNA while no variation was inherited from the father. Quantitative PCR suggested a paternally inherited deletion.

Targeted MPS with CovCop (Derouault et al., 2017) detected the deletion in every patient, either in a homozygous (patient F1-II1) or in a heterozygous state (F1-I1, F1-I2, F2-I1).

The SNP study showed that pathogenic haplotype at the *CYP24A1* locus was the same between the two families (Fig. 1).

Breakpoints study evidenced the same 10,924-bp long deletion in both families, from the end of exon 9 to the *CYP24A1* downstream region (hg19 chr20:52763705_52774628del) (Fig. 2).

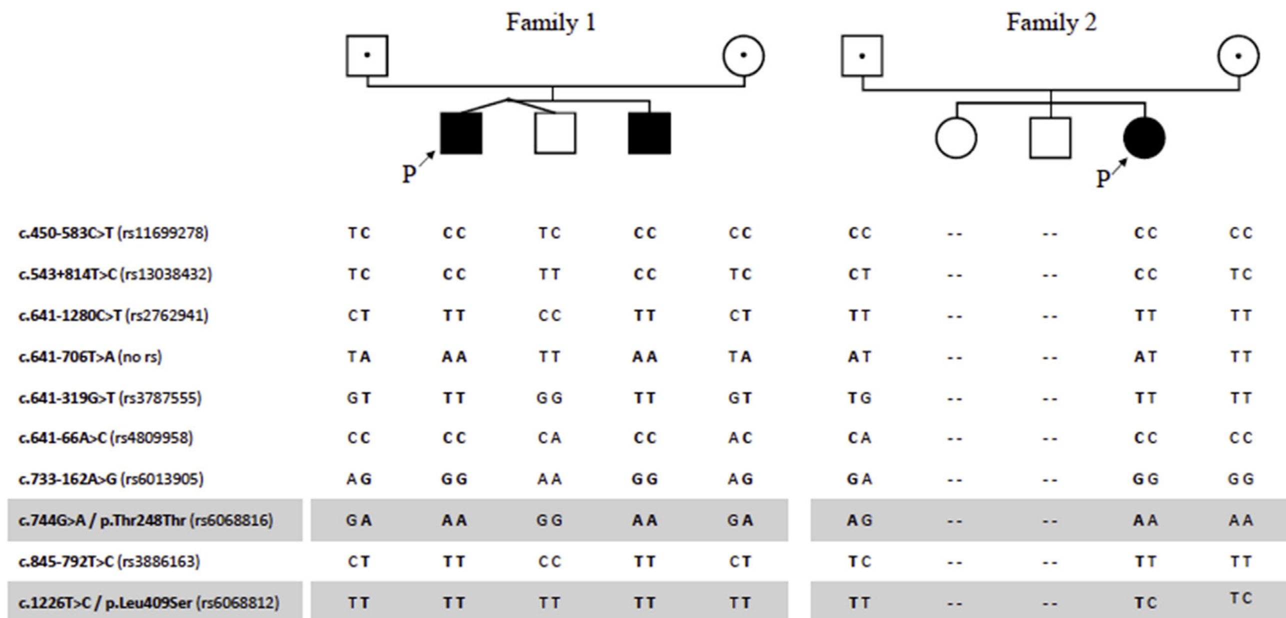
4. Discussion

In two unrelated families with IIH, a large *CYP24A1* deletion was characterized which might go undetected without adequate molecular diagnosis procedure.

The examination of the Database of Genomic Variants (DGV, http://dgv.tcag.ca/dgv/app/home) revealed several deletions at *CYP24A1* locus, ranging from 0.6 to 28 kb, with an estimate of the frequency of 0.01% (3/29,084) (14). Two similar deletions (nsv1063981 and esv2762085) were detected using SNP-array in an American patient with developmental delay and in a Swiss healthy man (Fig. 2) (Coe et al., 2014; Vogler et al., 2010). Their length (11,296 bp) was defined according to the array's probes' localization, which may explain the difference with our deletion's length. Unfortunately, no DNA sample from these patients was available to test this hypothesis. Allelic frequency of this particular deletion is currently unknown. It was identified with the same haplotype in the 3 unrelated heterozygous parents (F1-I1, F1-I2, F2-I1), which may suggest a founder effect (as both families shares common geographic origins in Normandy) and a significant frequency in general population. Thus, a special attention should be paid to CNV analysis during molecular diagnosis of IIH, as it might represent a significant part of *CYP24A1* molecular defects.

Intragenic deletion can easily remain undetected by Sanger sequencing when at least one primer targets a deleted sequence, leading to misdiagnosis, wrong interpretation of genetic data and wrong genetic counseling. In patients harboring the mutation in a homozygous state, the lack of PCR amplification draws attention, as illustrated in family 1. Conversely, in the case of a deletion in a heterozygous state, only one allele is amplified and sequenced, which leads to an incorrect diagnosis of homozygosity, as illustrated in family 2. Indeed, the lack of amplification of both allele of exon 9 explains why the p.Leu409Ser mutation appeared in a homozygous state in F2-II3.

Other techniques for CNV detection such as quantitative PCR, Multiplex Ligation Probe Analysis (MLPA) (targeted approachs), arrayCGH or SNP-array (pangenomic approachs) have been used to



SNP	Family 1					Family 2				
c.450-583C>T (rs11699278)	TC	CC	TC	CC	CC	CC	--	--	CC	CC
c.543+814T>C (rs13038432)	TC	CC	TT	CC	TC	CT	--	--	CC	TC
c.641-1280C>T (rs2762941)	CT	TT	CC	TT	CT	TT	--	--	TT	TT
c.641-706T>A (no rs)	TA	AA	TT	AA	TA	AT	--	--	AT	TT
c.641-319G>T (rs3787555)	GT	TT	GG	TT	GT	TG	--	--	TT	TT
c.641-66A>C (rs4809958)	CC	CC	CA	CC	AC	CA	--	--	CC	CC
c.733-162A>G (rs6013905)	AG	GG	AA	GG	AG	GA	--	--	GG	GG
c.744G>A / p.Thr248Thr (rs6068816)	GA	AA	GG	AA	GA	AG	--	--	AA	AA
c.845-792T>C (rs3886163)	CT	TT	CC	TT	CT	TC	--	--	TT	TT
c.1226T>C / p.Leu409Ser (rs6068812)	TT	TT	TT	TT	TT	TT	--	--	TC	TC

Fig. 1. Pedigree trees and haplotypes study. Nine single nucleotide polymorphisms (SNP) and one *CYP24A1* mutation c.1226T > C/p.Leu409Ser (rs6068812) were used to determine a common pathogenic haplotype. Grey boxes symbolize exonic SNP.

further genetic analysis in case of highly specific phenotype with negative sequencing, in various diseases (Ellingford et al., 2018). Accuracy of these techniques greatly depends on the number and localization of probes. More recently, specific algorithms to detect CNV among MPS data (whole genome sequencing (WGS), WES or targeted MPS) are being developed to improve the sensitivity of genetic analysis (Ellingford et al., 2018; Zhao et al., 2013). Our targeted MPS with CNV detection method based on CovCop (Derouault et al., 2017), a user friendly tool made to identify CNV among amplicons sequencing data, was able to detect the deletion in every patient including heterozygous patient.

In conclusion, instead of illustrating one new mutation, this report confirms the role of *CYP24A1* CNV in IIH. Physicians must keep in mind that a non-contributory *CYP24A1* coding sequence analysis (e.i. no mutation or a mutation in a heterozygous state) in a IIH patient may have two different meanings, either a truly negative result, which should lead to consider another mechanism of IIH (*SLC34A1* mutation), or a CNV which may be detected with an appropriate method. In that perspective, evaluation of 24,25-dihydroxyvitamin D (Kaufmann et al., 2014) and familial study are critical for a relevant interpretation of genetic data and to point patients who may harbor intragenic rearrangement.

Conflicts of interest

The authors declare no conflict of interest.

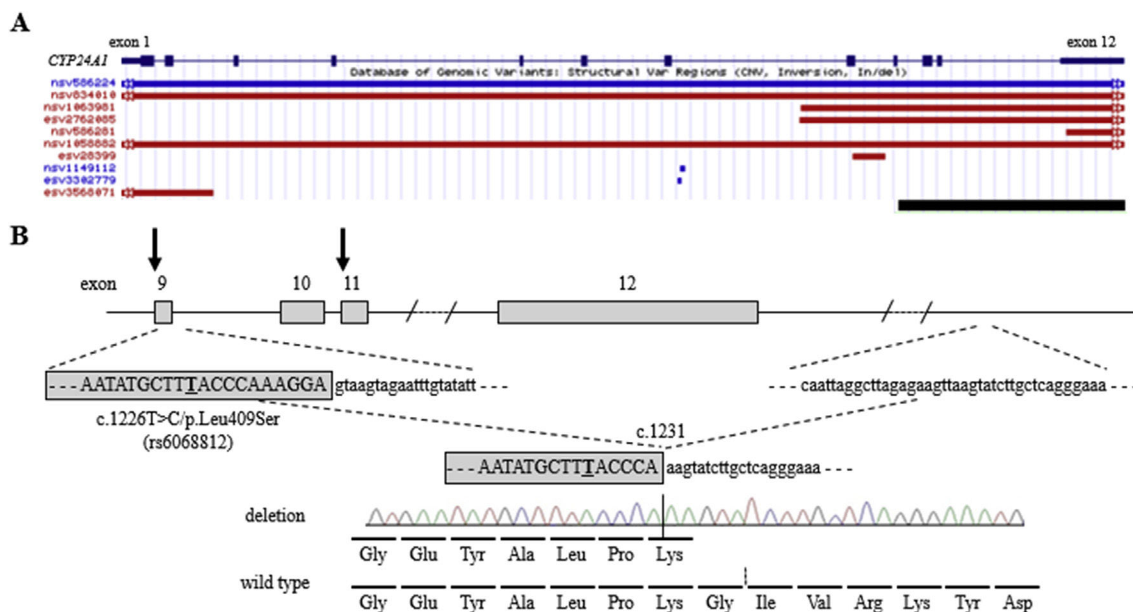


Fig. 2. (A) Schematic representation of the *CYP24A1* rearrangements indexed in Database of Genomic Variants (DGV) (http://dgv.tcag.ca/dgv/app/home). Blue lines are duplications, red lines are deletion. (B) Schematic representation of the *CYP24A1* deletion characterized through Sanger sequencing of the 595-pb fragment obtained from PCR with R14. Black arrows represent Taqman assays. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Acknowledgements

The authors would like to thank the patients and their families, Anne-Sophie Lia and the CovCop team, Christian Vogler and Candy Lee, Délégation de la Recherche Clinique et de l'Innovation (DRCI) - CHU de Caen APRIM - 16-048.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmg.2018.11.011.

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Etude de *VDR*, GC, *PDIA3* et *CYP3A4*

L'étude de la cohorte n'a révélé aucune variation d'intérêt dans les gènes *VDR*, *GC*, *PDIA3* et *CYP3A4* quel que soit le phénotype considéré.

Etude des gènes impliqués dans la fuite tubulaire de phosphate

L'objectif de ce travail était de rechercher des variations des gènes actuellement impliqués dans la réabsorption tubulaire de phosphate (*SLC34A1*, *SLC34A3* et *SLC9A3R1*) chez des patients présentant un phénotype compatible avec un déficit en vitamine D 24-hydroxylase sans mutation de *CYP24A1* identifiée.

Chevauchement phénotypique associé aux mutations de *CYP24A1*, *SLC34A1* et *SLC34A3* : étude d'une cohorte de patients avec hypersensibilité à la vitamine D

Overlapping phenotypes associated with CYP24A1, SLC34A1 and SLC34A3 mutations: a cohort study of patients with hypersensitivity to vitamin D

Cet article (soumission le15/09/2019 dans *Kidney International*) porte sur l'étude par MPS de la cohorte de 72 patients précédemment publiée et enrichie de 113 nouveaux patients, soit 185 patients au total avec suspicion de déficit en vitamine D 24-hydroxylase.

Outre l'identification de nouvelles variations potentiellement pathogènes dans les gènes *CYP24A1*, *SLC34A1* et *SLC34A3*, cette étude souligne le chevauchement phénotypique entre les maladies associées à ces loci, notamment par la description d'hypophosphatémie chez des patients avec mutation *CYP24A1*, par la mise en évidence de variations potentiellement pathogènes de *SLC34A1* ou *SLC34A3* chez des patients sans hypophosphatémie. Ces observations renforcent l'hypothèse émise par Schlingmann *et al.* d'une perturbation du métabolisme de la vitamine D secondaire à une anomalie du métabolisme du phosphate visant à maintenir une phosphatémie normale mais conduisant à une dérégulation de l'absorption intestinale de calcium.

Enfin, l'étude d'une famille particulière issue de cette cohorte suggère qu'un mécanisme de digénisme pourrait être responsable d'un phénotype d'hypersensibilité à la vitamine D (hétérozygotie *CYP24A1* et *SLC34A3*).

Overlapping phenotypes associated with *CYP24A1, SLC34A1* and *SLC34A3* mutations: a cohort study of patients with hypersensitivity to vitamin D

Running title: Genetic in hypersensitivity to vitamin D

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37 **Grant supporters**

38 Délégation Recherche Clinique et Innovation (DRCI) - CHU de Caen APRIM - 16-048

39 **Abstract**

40 Mutations in *CYP24A1* (vitamin D 24-hydroxylase) and *SLC34A1* (renal phosphate transporter
41 NPT2a) cause autosomal recessive Infantile Hypercalcemia type 1 and 2, illustrating links
42 between vitamin D and phosphate metabolism. Patients may present hypercalciuria and
43 alternate between chronic phases of normocalcemia with inappropriately high 1,25-$(OH)_2$D and
44 appropriately low PTH, and acute phases of hypercalcemia with suppressed PTH. Mutation in
45 *SLC34A3* and *SLC9A3R1* have been associated with phosphate wasting without hypercalcemia.

46 The aims of this study were to evaluate the frequency of mutations in these genes in patients
47 with a medical history suggestive of *CYP24A1* mutation to search for a specific pattern. Using

next generation sequencing, we screened for mutations in 185 patients with PTH levels < 20 pg/mL, hypercalcemia and/or hypercalciuria, and relatives.

Twenty-eight (15%) patients harbored biallelic mutations in *CYP24A1* (25) and *SLC34A3* (3), mostly with renal disease (lithiasis, nephrocalcinosis) (86%). Hypophosphatemia was found in 7 patients with biallelic mutations in *CYP24A1* and a normal phosphatemia was reported in 2 patients with biallelic mutations in *SLC34A3*. Rare variations in *SLC34A1* and *SLC34A3* were mostly of uncertain significance. Fifteen patients (8%) carried only one heterozygous mutation. Heterozygous relatives carrying *SLC34A1* or *SLC34A3* variation may present with biochemical changes in mineral metabolism. Two patients' genotype may suggest digenism (heterozygous variations in different genes). No variation was found in *SLC9A3R1*.

As no specific pattern can be found, patients with medical history suggestive of *CYP24A1* mutation should benefit from *SLC34A1* and *SLC34A3* analysis.

Key words: hypersensitivity to vitamin D – hypercalcemia – *CYP24A1*- phosphate wasting – *SLC34A1 – SLC34A3*

63 **INTRODUCTION**

64 Hypersensitivity to vitamin D (HVD) can be defined as an inability to regulate vitamin D levels
65 which lead to calcium homeostasis deregulation, especially in case of vitamin D
66 supplementation[1]. This phenotype was classically associated with idiopathic infantile
67 hypercalcemia (IIH), a condition firstly described in the early fifties [2]. Children present with
68 non-specific signs of hypercalcemia (failure to thrive, vomiting, polyuria and polydipsia) with
69 adapted low parathormone (PTH) but therefore with and high 1,25-$(OH)_2D$, suggesting
70 deregulation of vitamin D metabolism.

71 In 2011, mutations in *CYP24A1* encoding the vitamin D 24-hydroxylase were identified as the
72 cause of Infantile Hypercalcemia type 1 (IH1) (MIM 143880) with HVD phenotype [3,4]. This
73 enzyme is responsible for 1,25-$(OH)_2D$ and 25-OH-D inactivation [5–7]. A high 25-OH-D_3:24,25-
74 $(OH)_2D_3$ ratio (substrate: product ratio, R ratio), which directly reflects the enzymatic defect,
75 has been specifically associated with this condition and proposed as a screening tool [8–10]. The
76 clinical spectrum has been broadened to older children, teenagers and adults with
77 nephrocalcinosis, renal stones and chronic high fluctuant serum and urine calcium with
78 decreased serum PTH [9,11–15]. Patients present with a lifelong susceptibility to acute
79 hypercalcemia and hypercalciuria, especially in case of vitamin D supplementation [16] and
80 sunshine exposure [17].

81 In 2016, mutations in *SLC34A1*, encoding the renal phosphate transporter NPT2a, were reported
82 as the cause of Infantile Hypercalcemia type 2 (MIM 616963) with renal phosphate wasting
83 and hypophosphatemia [18]. A few cases have been published thereafter with clinical phenotype
84 similar to type 1 [19–21]. These observations suggest that phosphate wasting disorders can lead to
85 HVD.

86 Other genes have been associated with renal phosphate wasting. Mutations in *SLC34A3*
87 encoding the renal phosphate transporter NPT2c have been associated with autosomal recessive
88 hypophosphatemic rickets (ARHR) with hypercalciuria (MIM 241530) [22,23]. Its phenotype also
89 includes biochemical features of HVD (low PTH, high serum 1,25-$(OH)_2D$, high urine calcium
90 with renal stones and nephrocalcinosis) and normal to low FGF23 levels. Furthermore, two
91 patients with idiopathic hypercalciuria and biallelic variations in *SLC34A3* were described
92 suggesting a potential role of *SLC34A3* in HVD phenotype without bone sign [24]. Lastly,
93 *SLC9A3R1*, encoding NHERF1 (sodium-proton exchanger regulatory factor 1) protein was
94 previously associated with autosomal dominant phosphate wasting [25,26]. This protein is
95 implicated in targeting of NPT2a to the apical membrane of the proximal tubular cell and
96 control of its retrieval by PTH [27].

97 We have previously published a cohort of 72 patients (1-72, Table S1) with HVD tested for
98 *CYP24A1* mutation[9]. In the present study, we aimed to evaluate the respective contribution of
99 abovementioned genes involved in phosphate wasting in this cohort enriched with 113 new
100 patients. Our objective was also to search for a diagnostic algorithm to guide candidate gene
101 testing in patients with features of HVD.

102 **PATIENTS & METHODS**

103 ***Patients***

104 Over a 5 years period, we enrolled for molecular analysis 185 patients (index cases) presenting
105 with PTH levels <20 pg/mL (2nd generation PTH assay were considered, normal values: 15-65
106 pg/mL) and a medical history of acute or chronic hypercalcemia (>2.6 mmol/L) and/or chronic
107 hypercalciuria. Recruitment was national thanks to the network of the National Center for rare
108 diseases of calcium and phosphate metabolism.

109 After genetic counseling, relatives of patients carrying biallelic variations were studied for
110 genetic screening. Clinical and biochemical data were available in 17 relatives.
111 Written informed consent was obtained from the patients and/or their parents for retrospective
112 collection of clinical and laboratory data, and for DNA collection to conduct molecular studies.

113 ***Biochemical and clinical parameters***

114 Data on clinical symptoms were collected retrospectively using records from hospitals or
115 primary care physicians.
116 Data on biochemical parameters (calcium and phosphate in serum and urine, renal function:
117 creatinine and estimation of glomerular filtration rate with CKD-EPI for adults[28], PTH and
118 vitamin D levels) were collected retrospectively using the same procedure, and thus correspond
119 to a collection of different methods from many clinical chemistry departments. Approximate
120 normal values for 1,25-$(OH)_2D$ were used as follow considering the use of different assays
121 inherent in such retrospective study: 65-134 pmol/L for children and 60-108 pmol/L for adults
122 [29]. As most of routine assays for vitamin D cannot discriminate between vitamin D_3 and D_2, we
123 use the term "vitamin D" to describe these measurements. As serum phosphate decreases from
124 birth to adulthood, values were expressed as Z-scores to avoid the effects of the age-related
125 variations of reference values [24,30,31].
126 Routine biochemical assays were performed at the time of the diagnosis of acute hypercalcemia,
127 or during follow up after normalization of the calcium level.
128 For urinary calcium:creatinin ratio, the different cutpoints of normal level are as follow: adults
129 <0.33; children under 6 months <2.4; children 7-12 months <1.7; children 1-5 years <1.1 and
130 children above 5 years <0.7.

131 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) analysis was performed as
132 previously described using 100 µL of serum. Results are expressed as the molar ratio of 25-

133 OH-D_3: 24,25-$(OH)_2D_3$ (R ratio) [8]. Values under 25 indicated no defect in 25-OH-D_3-24-
134 hydroxylase activity and were considered as normal.
135 Medullary nephrocalcinosis was assessed by renal ultrasonography (echogenic renal pyramids).
136 Nephrolithiasis was defined as kidney stones on ultrasonography or a medical history of
137 iterative renal colic.

138 ***Molecular analysis***

139 Genomic DNA was extracted from whole blood samples with QuickGene DNA Whole Blood
140 kit (Kurabo Industries LTD, Osaka, Japan) and QuickGene-610L extraction system (Fujifilm
141 LTD, Singapore, Republic of Singapore).
142 Coding exons and and flanking regions of *CYP24A1* (NM_000782.4), *SLC34A1*
143 (NM_003052.4), *SLC34A3* (NM_001177317.1) and *SLC9A3R1* (NM_004252.4) genes were
144 studied by Massively Parallel Sequencing (MPS) using a custom Ampliseq library on the Ion
145 Torrent Personal Genome Machine (PGM) (ThermoFisher Scientific, Waltham, Massachusetts,
146 USA). Reads were aligned against the reference sequences and variants were annotated using
147 IonSuit (ThermoFisher Scientific, Waltham, Massachusetts, USA) and Nextgene
148 (SoftGenetics, State College, Pennsylvania, USA). A read depth (RD)-based approach was used
149 to detect copy number variation (CNV) from targeted MPS data using the CovCop tool [32].
150 Sequences with coverage <30X and variations identified with MPS were studied with Sanger
151 sequencing using Big Dye® Terminator v1.1 Cycle Sequencing kit (ThermoFisher Scientific,
152 Waltham, Massachusetts, USA) on a ABI 3500 Sequencer (ThermoFisher Scientific, Waltham,
153 Massachusetts, USA).
154 Variations of sequence were searched on Varsome database. Interpretation was based on
155 standards and guidelines of the American College of Medical Genetics[33]. Pathogenicity
156 prediction programs PolyPhen-2, Align-GVGD, MutationTaster and SIFT were considered for
157 in silico analysis. Allelic frequencies were evaluated through the examination of GnomAD

158 database. For research purposes, variations classified as of uncertain significance (III) were
159 considered apart from mutations (i.e. likely pathogenic (IV) and pathogenic (V) variations) as
160 some may represent pathogenic mutations.

161 ***Statistical analysis***

162 Data from patients with class IV and V variations were used to compare biochemical profiles.
163 Statistical analyses were made using Prism 7 (GraphPad Software Inc.). For each biochemical
164 parameter, differences between groups were calculated using the Mann and Whitney
165 nonparametric U test. A two-tailed p value <0.05 was considered statistically significant.
166 Kruskal-Wallis test was used for multiple group comparison (one-way nonparametric
167 ANOVA).

168 Sex was not considered a factor in the statistical analysis of the data.

169 Chi-squared test was used to compare the allelic frequency of recurrent variations in our cohort
170 and in the general population (estimated from data of GnomAD database).

171 **RESULTS**

172 ***Patients***

173 We enrolled 185 patients (88 males (47%) and 97 females (53%)), including 150 children (81%)
174 (range: 1 day – 16 years; mean 2.3 years) and 35 adults (19%) (range: 18-81 years; mean 42
175 years). Familial occurrence was recorded in 34 patients (18%).

176 ***Molecular analysis: variations*** (Table 2)

177 The frequency and the criteria in favor of deleteriousness of each variant are presented in Table
178 2.

179 We identified 20 mutations in *CYP24A1*, 1 mutation in *SLC34A1* and 6 mutations in *SLC34A3*.
180 Adding class III variations, the number of variations rose to 21 variations in *CYP24A1*, 9
181 variations in *SLC34A1* and 15 variations in *SLC34A3*.

182 We described 2 new missenses pathogenic variations c.980C>T/p.(Ala327Val) and
183 c.1193T>C/p.(Leu398Pro) in patients with elevated R ratio.

184 The recurrent variation in *SLC34A1* c.272_292del/p.Val91_Ala97del (REC DEL in Table S1)
185 was identified in 10 patients (Table 1). In our cohort, its allelic frequency is 2.7%. A Chi-
186 squared test showed that this frequency was not statistically different from the allelic frequency
187 found in general population (1.696 % in general population, 2.537% in European non-Finnish
188 population according to GnomAD Exomes database).

189 ***Molecular analysis: genotype***

190 Patients were classified into different groups according to their genotype (gene with variation,
191 biallelic, i.e. homozygous or compound heterozygous, or monoallelic, i.e. heterozygous) (Table
192 1).

193 Depending on class III variations, we identified biallelic variations in 28-36 patients (15-19%)
194 and monoallelic variations in 15-25 patients (8-14%) (heterozygous status) and no variation
195 was found in 122-142 patients (66-77%) patients. Variations in *CYP24A1* (biallelic as well as
196 monoallelic) are more frequently found (18-19%) followed by those in *SLC34A1* (1-8%) and
197 *SLC34A3* (5-6%). No variation was found in *SLC9A3R1*.

198 ***Digenic patients***

199 Two patients presented with one heterozygous class III *SLC34A3* and a second variation in
200 *SLC34A1* (recurrent deletion) or *CYP24A1* (the recurrent pathogenic variation p.Leu409Ser).
201 Especially, we reported 2 children of a non-consanguineous healthy couple who had
202 experienced symptomatic neonatal hypercalcemia with similar biochemical profile (Figure 2).
203 Both carried a *SLC34A3* variation of uncertain significance inherited from their father and a

CYP24A1 recurrent mutation inherited from their mother. The father presented with vertebral non-traumatic fracture; the mother was healthy.

Phenotype of patients with biallelic variation

Biallelic variations were found in higher frequency in adult patients (45-51%; n= 16-18/35) than in children (8-12%; n= 12-18/150), most aged over 2 years (Table 1).

In patients with nephrocalcinosis (n=55; 44 children and 11 adults), we identified 18 (33%) patients with biallelic class IV or V variations, mostly in *CYP24A1* (n=25/28) and also in *SLC34A3* (n=3/28); 4 adults with biallelic *CYP24A1* mutations presented chronic kidney disease (CKD-EPI<60mL/min). In patients with renal lithiasis (n=28; 18 children and 10 adults), we identified 6 (21%) patients with biallelic mutations in *CYP24A1*, mostly adults (n=5/6). Of note, *CYP24A1* biallelic mutations were also found in 4 adult patients with medical history of hypercalcemia without nephrocalcinosis nor renal stones.

One patient was referred at the age of 5 for hypercalciuria and nephrocalcinosis, normal serum calcium and low PTH. At the age of 10, bowing of the lower limbs was evocative of rickets. He harbored a homozygous *SLC34A3* mutation inherited from his consanguineous parents. His mother and his two brothers with the same mutation in a heterozygous state also presented with nephrocalcinosis without any signs of bone disease.

Including class III variations, we identified 3 more patients with biallelic variations in *SLC34A3* and nephrolithiasis (n=1) or nephrocalcinosis (n=2) and 4 patients with biallelic *SLC34A1* variations, 2 of them (patients 103 and 170) having nephrocalcinosis detected as hyperechogenic kidney *in utero* [21].

Hypophosphatemia (serum phosphate Z-score < -2) was reported in 7 patients (27-28%) with *CYP24A1* biallelic mutations and 1 patient with *SLC34A3* biallelic mutations (Figure 1, Table S1).

228 Serum calcium in patients with biallelic *SLC34A3* mutation was lower than in patients with
229 biallelic *CYP24A1* mutation (p=0.0123*). Serum 25-OH-D in patients with biallelic *CYP24A1*
230 mutation was higher than in patients with biallelic *SLC34A3* mutation (p=0.0139*). There was
231 no other statistically significant difference between these two groups of patients, even if serum
232 phosphate (SD) tended to be lower in patients with biallelic *SLC34A3* mutation.

233 ***Patients with mutation in a heterozygous state***

234 Mutations in a heterozygous state were found in patients with renal stones (n=6),
235 nephrocalcinosis (n=4) and without renal complications (n=5), mostly in *CYP24A1* (n=9) and
236 *SLC34A3* (n=5).
237 Including class III variations, we identified 11 patients with *SLC34A1* variations (7 of them
238 carrying the recurrent c.272_292del/p.Val91_Ala97del) and 3 with a *SLC34A3* variation.
239 Among this 14 patients with heterozygous class III variation, 6 had nephrocalcinosis, 1
240 nephrolithiasis and 7 no renal disease. Especially, 3 patients with heterozygous
241 c.272_292del/p.Val91_Ala97del had nephrocalcinosis.
242 Serum PTH of probands with *SLC34A3* mutations in a heterozygous state is higher than serum
243 PTH in patients with biallelic *SLC34A3* and *CYP24A1* mutations (p=0.0357* and p=0.0003***
244 respectively). Biochemical profile of relatives and probands with *SLC34A3* mutations in a
245 heterozygous state did not differ statistically, although PTH tended to be higher and 25-OH-D
246 and 1,25-$(OH)_2D$ tended to be lower in relatives. We observed typical HVD biochemical
247 features in *SLC34A3* relatives' group, namely hypercalciuria in 8/11 cases, low serum PTH in
248 6/11 cases, high 1,25-$(OH)_2D$ in 9/11 cases. Similarly, 2/4 relatives carrying *SLC34A1* class III
249 variation presented biochemical changes, 1 having a high urinary calcium excretion and 1
250 having hypophosphatemia (<-2 Z-score).

251 **DISCUSSION**

252 This report presents extensive clinical, biochemical and molecular data on a large cohort of
253 patients with HVD phenotype and suspicion of IH1. Main messages for the physicians are the
254 following: i) Mutations in genes associated with renal phosphate wasting can be found with a
255 lower frequency than loss-of-function mutations in *CYP24A1* and digenism may represent a
256 rare mechanism of HVD. ii) Our data indicate that hypophosphatemia is not specific to
257 phosphate wasting defects, also observed in *CYP24A1* mutation, explaining a wide phenotypic
258 overlap.

259 In our population, we found biallelic variations in *CYP24A1*, *SLC34A1* and *SLC34A3*, in
260 accordance with autosomal recessive inheritance, in 28-36 patients (15-19%), but no mutation
261 in *SLC9A3R1*, suggesting that mutations in this latter gene do not cause HVD phenotype. We
262 observed a majority of biallelic mutations in *CYP24A1*, in accordance with inclusion criteria
263 (medical history of hypercalcemia and/or hypercalciuria and low PTH levels). We found a high
264 number of variations of uncertain significance in *SLC34A1* and *SLC34A3* (17/24) with
265 difficulties to interpret their pathogenicity in a context of routine molecular diagnosis. For
266 example, the signification of the recurrent c.272_292del / p.Val91_Ala97del variation
267 previously described and associated to IH2 could still be questioned. Its allelic frequency in
268 general population is high and similar in our cohort, suggesting a polymorphism rather a loss
269 of function mutation. Although its biological activity was preserved, it was reported as a
270 pathogenic variation responsible for cellular localization defect [18,37]. However, its association
271 with another variation in *SLC34A1* in several patient [18] cannot exclude some effect in terms of
272 pathogenicity, maybe as an hypomorphic allele. Interpretation is made more complex by the
273 high number of rare variations in these genes reported in a heterozygous state in online
274 population databases (i.e. more than 700 *SLC34A3* variations in GnomAD). In vitro functional
275 studies may seem likely difficult in a context of routine diagnosis, justifying the need for
276 specific clinical or biochemical criteria and the collection of molecular data.

277 Most of patients with biallelic variation present with renal complications of chronic
278 hypercalciuria, renal stones (6/28) or nephrocalcinosis (18/28) and were adults or children more
279 than 2 years old (24/28). The lower diagnosis rate observed in children (8-12% versus 45-51%)
280 suggest that most of the children in our cohort could present transient HVD due to the initial
281 prematurity in renal function observed during the early infancy [3]. Patients with biallelic
282 variation in *CYP24A1* and *SLC34A1* presented with a great phenotypic overlap, easily
283 distinguished with 25-OH-D_3:24,25-$(OH)_2D_3$ ratio (substrate: product ratio, high in patients
284 with mutation in *CYP24A1*, normal in patients with *SLC34A1*) which directly reflects the
285 vitamin D 24-hydroxylase defect [8–10]. The presence of prenatal hyperechogenic kidneys in 2
286 patients of this cohort with *SLC34A1* class III variations and 2 patients in the literature [21]
287 suggests a more precocious disease of the urinary tract. To the best of our knowledge, no
288 patients with prenatal hyperechogenic kidneys and *CYP24A1* mutation were reported in the
289 literature or observed in our patients. Mutations in *SLC34A3* are responsible for ARHR, with
290 characteristic high urine calcium and serum 1,25-$(OH)_2D$, but hypercalcemia in these patients
291 was not documented up to date. Among patients with biallelic *SLC34A3* variations, we report
292 2 patients with fortuitously discovered hypercalcemia, a patient (n°152) with initial clinical
293 feature of HVD whose clinical presentation evolved into classic ARHR (with short stature and
294 legs bowing), a patient (n°88) with HVD with nephrocalcinosis without bone disease nor
295 hypophosphatemia and normal height. These observations widen the phenotypic spectrum of
296 *SLC34A3* mutations to HVD. Whether the HVD presentation correspond to a sustainable mild
297 form of phosphate tubular reabsorption defect or to a precocious presentation of ARHR requires
298 further investigation.

299 In our cohort, only 2 patients with biallelic variation in *SLC34A3* (class V) or *SLC34A1* (class
300 III) presented with hypophosphatemia. Thus, most of them do not present with biochemical
301 serum profile suggestive of a phosphate wasting disorder *prima facie*, including 2 patients with

302 biallelic *SLC34A3* pathogenic variations. Similarly, an approximate third (5/16) of patients do
303 not presented with hypophosphatemia in the IIH cohort published by Schlingmann et al. [18] and
304 patients with biallelic mutations in *SLC34A3* and normal serum phosphate have also been
305 published [24]. More surprisingly, 27-28% of our patients with *CYP24A1* biallelic mutation
306 presented with hypophosphatemia. Taken together, these observations enlighten the role of
307 FGF23 in HVD [18]. Indeed, FGF23 is a key regulator of phosphate and vitamin D homeostasis,
308 up-regulated by serum phosphate and 1,25-$(OH)_2D$ [39,40]. In turn, its fixation on FGF receptors
309 induces the retrieval of renal phosphate transporters (responsible for phosphate tubular
310 reabsorption) and decrease the expression of vitamin D 1α-hydroxylase (*CYP27B1*) [41]. In IH1,
311 we suggest that reduced clearance of 1,25-$(OH)_2D$ up-regulates FGF23 production and
312 consequently leads to phosphate wasting and hypophosphatemia. In contrast, in NaPiIIa/NPT2a
313 / NaPiIIc/NPT2c defect, renal phosphate wasting is responsible for decrease in serum phosphate
314 which in turn down-regulates FGF23. Thanks to 1α-hydroxylase activation, the increase in
315 1,25-$(OH)_2D$ favors phosphate and calcium intestinal absorption. The negative feedback
316 exerted by the resulting increase in serum calcium is supported by the low serum PTH. Such
317 mechanism had previously been proposed and corroborated by murine Slc34a1-/- studies,
318 illustrating the crucial role of FGF23 as a key regulator of vitamin D metabolism [18]. Case reports
319 have thus suggested that supplementation with oral phosphate could contribute to correct
320 calcium metabolism in patients with *SLC34A1* mutation [18].

321 In 15-25 patients, we identified variations in a heterozygous state, which represent a diagnosis
322 issue in a context of a classic autosomal recessive disease. Indeed, we cannot exclude a second
323 variation situated in non-coding sequence for instance or in another unknown gene, as the role
324 of environmental factors or modifier genes. Although an autosomal dominant trait (7) has been
325 proposed for heterozygous *CYP24A1* loss-of-function mutation [34], our previous work showed
326 that heterozygous patients (proband as well as relatives) have normal 24-hydroxylase activity

327 [9,13]. We observed *SLC34A3* heterozygous relatives presenting with a typical HVD phenotype
328 which obviously had not been diagnosed as HVD patients. Similarly, it was suggested that
329 *SLC34A1* heterozygous mutation could be a risk factor for kidney stone disease [18,35] and
330 heterozygous relatives may have abnormal biochemical data. It is likely that heterozygous
331 phenotype depends on additional factors, including genetics. Indeed, families in which the
332 probands harbor a variation in two different genes suggest that heterozygous variations in
333 *CYP24A1*, *SLC34A1* and *SLC34A3* may have an impact on vitamin D metabolism with a
334 deleterious synergistic effect as previously proposed [36], as illustrated by the lower PTH level in
335 these patients compare to their heterozygous parents.

336 Unfortunately, unlike genetic data obtained from the same procedure, the data collected in this
337 retrospective cohort may include bias due to the aggregation of biochemical results from
338 various assays and routine medical laboratories, which can impact the interpretation of 1,25-
339 $(OH)_2D$ particularly. Appropriate exploration of phosphate metabolism including serum FGF23
340 evaluation would be of great interest to further characterize this pathophysiologic mechanism
341 in HVD. Lastly, several patients without mutation (n=24) presented with hypophosphatemia
342 suggesting the existence of other different mechanisms of calcium, vitamin D and phosphate
343 metabolism deregulation.

344 **CONCLUSION**

345 Without specific biochemical profile to one or another gene, we recommend analyzing
346 *SLC34A1* and *SLC34A3* during genetic exploration of HVD in patients without vitamin 24-
347 hydroxylase deficiency (normal *CYP24A1* sequencing and normal R ratio), even in adults.

348 This is far the more important in the actual growing context of personalized medicine, as
349 different therapies have been proposed (hydrochlorothiazide, ketoconazole and fluconazole,
350 phosphate oral supplementation).

Authors contributions

MA and KML designed the study; BC and CN realized molecular study; MA, RN, MH interpreted molecular study; MA, LS, BJ, BP, LA, and NM collected the clinical and biochemical data; KM and JG realized LC-MS/MS studies; MA analyzed the data, drafted the paper and made all tables and figures; KML, BJ, LS, JG and KM revised the paper; all authors approved the final version of the manuscript.

Acknowledgments

Authors acknowledge the patients and their families, all the physicians who collaborate with our center and Délégation Recherche Clinique et Innovation (DRCI) - CHU de Caen for its financial support (APRIM - 16-048).

Disclosure Summary

The authors have nothing to disclosure.

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574

575 <u>Legends:</u>

576 **Table 1:** Genotype in the cohort.

577 **Table 2:** Description of genetic variations which were identified in *CYP24A1*, *SLC34A1* and

578 *SLC34A3*.

579 f gnomAD: allelic frequency reported in exome gnomAD database; f all max (pop): maximum

580 allelic frequency reported in exome gnomAD database (population with maximum allelic

581 frequency); hmz: number of subjects carrying the variation in a homozygous state reported in

582 exome gnomAD database; UTR: untranslated region.

583

584 **Figure 1:** Biochemical data according to genotype: serum calcium (mmol/L), serum

585 phosphate (SD), PTH (pg/mL) 25-OH-D (nmol/L) and 1,25-$(OH)_2$D (pmol/L). Black dot:

586 patients with biallelic *CYP24A1* mutations. Grey dot: patients with heterozygous *CYP24A1*

587 mutations. Black triangle: patients with biallelic *SLC34A3* mutations. Grey triangle: patients

588 with heterozygous *SLC34A3* mutations. Grey diamond: patients with heterozygous *SLC34A1*

589 mutations. White square: patients without variation.

590 Serum calcium dot lines: normal values 2.2-2.7 mmol/L. Serum phosphate dot lines: -2-+2SD.

591 Serum PTH dot line: 15 pg/mL. 25-OH-D dot line: 75 nmol/L. 1,25-$(OH)_2$D dot and hyphen

592 lines: approximative normal values for children and adults.

593 * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.01$

594 **Figure 2: Pedigree tree of a family with suspected mechanism of digenism.** Both children

595 shared a biochemical profile evocative of HVD. Both parents shared a biochemical profile with

596 normal serum calcium, high 1,25-$(OH)_2$D, low PTH and a normal 25-OH-D evocative of mild

597 HVD.

598

599 **Table S1:** Raw dataset.

Table 1

	IV / V			III / IV / V		
	children	adults	total	children	adults	total
No variation	126	16	142 (77)	110	12	122 (66)
Biallelic variation	12	16	28 (15)	18	18	36 (19)
CYP24A1	9	16	25 (14)	10	16	26 (14)
SLC34A1	0	0	0 (0)	3	1	4 (2) [2]
SLC34A3	3	0	3 (2)	5	1	6 (3)
Monoallelic variation	12	3	15 (8)	20	5	25 (14)
CYP24A1	7	2	9 (5)	6	2	8 (4)
SLC34A1	1	0	1 (1)	9	2	11 (6) [7]
SLC34A3	4	1	5 (3)	5	1	6 (3)
Digenic patients	0	0	0 (0)	2	0	2 (1) [1]
TOTAL	150	35	185	150	35	185

Table 2

gene	variation type	rs	f gnomAD	hmz	f all max (pop)	exon	c.	p.	Pathogenic impact	benign impact	Variants class ACMG	Publication
CYP24A1	frameshift	774432244	0.0000626	0	0.0000626 (European non Finnish)	exon 1	c.62del	p.(Pro21Argfs*8)	PVS1, PM2, PM3, PP3		V	15
	frameshift	-	0.000004314	0	0.0001922 (other)	exon 1	c.233del	p.(Gly78Valfs*22)	PVS1,PM2,PM3, PP3		V	
	in frame	777676129	0.0005445	1	0.001065 (European non Finnish)	exon 2	c.427_429del	p.(Glu143del)	PS3, PS4, PM3, PM4, PP5		V	15
	missense	139763321	0.0001585	0	0.001827 (Ashkenazi Jewish)	exon 2	c.443T>C	p.(Leu148Pro)	PM3, PP1, PP3, PP5		IV	15
	nonsense	14718997603	0.000004063	0	0.00003249 (South Asian)	exon 3	c.464G>A	p.(Trp155*)	PVS1, PM2, PP3		V	15
	missense	-	-	-	-	exon 6	c.758T>A	p.(Met253Lys)	PS3, PM2, PM3, PP3		IV	15
	missense	-	-	-	-	exon 7	c.965A>C	p.(Glu322Ala)	PS3, PM2, PM5, PP3		IV	15
	missense	1224687481	0.00003236	0	0.0001152 (African)	exon 7	c.980C>T	p.(Ala327Val)	PS3, PM2 ,PM3, PP3		IV	-
	missense	552310427	0.00007715	0	0.001117 (Ashkenazi Jewish)	exon 7	c.989C>T	p.(Thr330Met)	PS3, PM2 ,PM3, PP3		IV	15
	frameshift	-	-	-	-	exon 8	c.1003dup	p.(Leu335Profs*11)	PVS1, PM2, PM3, PP3		V	15
	missense	-	-	-	-	exon 8	c.1138T>C	p.(Cys380Arg)	PS3, PM2 ,PM3, PP3		IV	15
	missense	114368325	0.0006825	1	0.001399 (European Finnish)	exon 9	c.1186C>T	p.(Arg396Trp)	PS3, PS4 ,PM3, PP3, PP5		V	15
	missense	143934667	0.00007317	0	0.0001523 (European non Finnish)	exon 9	c.1187G>A	p.(Arg396Gln)	PS4, PM2, PM3, PM5, PP3		V	15
	missense	-	-	-	-	exon 9	c.1193T>C	p.(Leu398Pro)	PS3,PM2,PM3		IV	
	frameshift	-	-	-	-	exon 9	c.1206del	p.(Val403Phefs*15)	PVS1, PM2, PM3, PP3	BP4	V	15
	missense	6068812	0.0007687	0	0.001371 (European non Finnish)	exon 9	c.1226T>C	p.(Leu409Ser)	PS3, PS4, PM3, PP3, PP5		V	15
	missense	374292194	0.00004469	0	0.0001825 (other)	exon 10	c.1315C>T	p.(p.Arg439Cys)	PS3, PM2, PP3		IV	15
	missense	748429181	0.000008123	0	0.00001791 (European non Finnish)	exon 10	c.1366G>C	p.(Gly456Arg)	PM2, PP3		III	15
	nonsense	988715134	0.000008123	0	0.00002978 (Latino)	exon 10	c.1396C>T	p.(Arg466*)	PVS1, PM2, PP3		V	
	frameshift	-	-	-	-	exon 10	c.1406_1407del	p.(Glu469Alafs*22)	PVS1, PM2, PM3		V	15
	large deletion	-	-	-	-	exon 10-12	g.52763705_52774628del	-	-	-	V	15
SLC34A1	in frame	876661296	0.01696	36	0.02726 (European Finnish)	exon 4	c.272_292del	p.(Val91_Ala97del)	PS3, PM3, PM4, PP5	BS2	III	18
	missense	-	-	-	-	exon 6	c.578T>A	p.(Ile193Asn)	PM2, PM3, PP3		III	
	splicing ?	200095793	0.00006499	0	0.0002599 (South Asian)	intron 9	c.1006+1G>A	p.?	PVS1, PS3, PM2, PP3, PP5		V	18
	missense	369749329	0.00008971	0	0.00008971 (European non Finnish)	exon 12	c.1352C>T	p.(Thr451Ile)	PM2, PP3		III	
	splicing ?	376448083	0.000354	0	0.0006564 (European non Finnish)	intron 12	c.1416+3G>A	p.?		BP4	III	

	splicing ?	202081023	0.0005237	0	0.0008868 (European non Finnish)	intron 12	c.1416+5G>A	p.?	PP5	BP6	III	18
	missense	756685605	4.07e-06	0	0.000008967 (European non Finnish)	exon 13	c.1466A>G	p.(Tyr489Cys)	PM2, PP3		III	
	missense	-	-	-	-	exon 13	c.1588G>A	p.(Val530Met)	PM2		III	
	missense	756519888	0.000008126	0	0.000008957 (European non Finnish)	exon 13	c.1645G>A	p.(Gly549Arg)	PM2		III	
SLC34A3	deletion	532224704	0.000965	0	0.004758 (African)	exon 4	c.195_215del	p.(Arg65_Gly71del)	PM4	BP6	III	
	splicing ?	201293634	0.00003666	0	0.00007199 (European Finnish)	intron 4	c.304+2T>C	p.?	PVS1, PM2, PP3, PP5		V	22
	missense	200536604	0.00004483	0	0.00008941 (Latino)	exon 6	c.496G>A	p.(Gly166Ser)	PM2, PP3		III	
	frameshift	-	-	-	-	exon 7	c.578_579insT	p.(Val194Glyfs*28)	PVS1, PM2	BP4	IV	
	deletion	-	1.31e-05	0	0.0001961 (other)	intron 9	c.925+20_926-48del	p.?	PVS1, PS3, PM2, PM3, PP5		V	23, 33
	missense	1405547154	0.00001688	0	0.00004061 (South Asian)	exon 10	c.926G>A	p.(Cys309Tyr)	PM2		III	
	frameshift	1473689787	6.56e-05	0	0.0001355 (European Finnish)	exon 10	c.944del	p.(Gly315Alafs*28)	PVS1, PM2	BP4	III	
	frameshift	-	-	-	-	exon 10	c.1055_1058dup	p.?	PVS1, PM2, PM3		V	
	frameshift	-	-	-	-	exon 10	c.1058_1065dup	p.?	PVS1, PM2		IV	
	missense	748862410	0.00002149	0	0.00004769 (European non Finnish)	exon 11	c.1198G>C	p.(Val400Leu)	PM2, PM3		III	
	missense	771932709	0.00002827	0	0.00002827 (European non Finnish)	exon 11	c.1207A>G	p.(Met403Val)	PM2	BP4	III	
	missense	532292902	0.00006341	0	0.0001889 (other)	exon 11	c.1208T>G	p.(Met403Arg)	PM2, PM3, PP3		III	
	missense	775653752	0.000004093	0	0.000009047 (European non Finnish)	exon 12	c.1283C>T	p.(Ala428Val)	PM2, PP3		III	
	missense	772211127	0.0000165	0	0.00006619 (African)	exon 13	c.1369G>A	p.(Gly457Ser)	PM2, PM3, PP5		III	24
	missense	149389629	0.0001118	0	0.0002501 (European non Finnish)	exon 13	c.1462G>C	p.(Ala488Pro)	PM3, PP3		III	

ACMG classification: III: variation of uncertain significance ; IV: likely pathogenic variation ; V : pathogenic variation

Figure 1

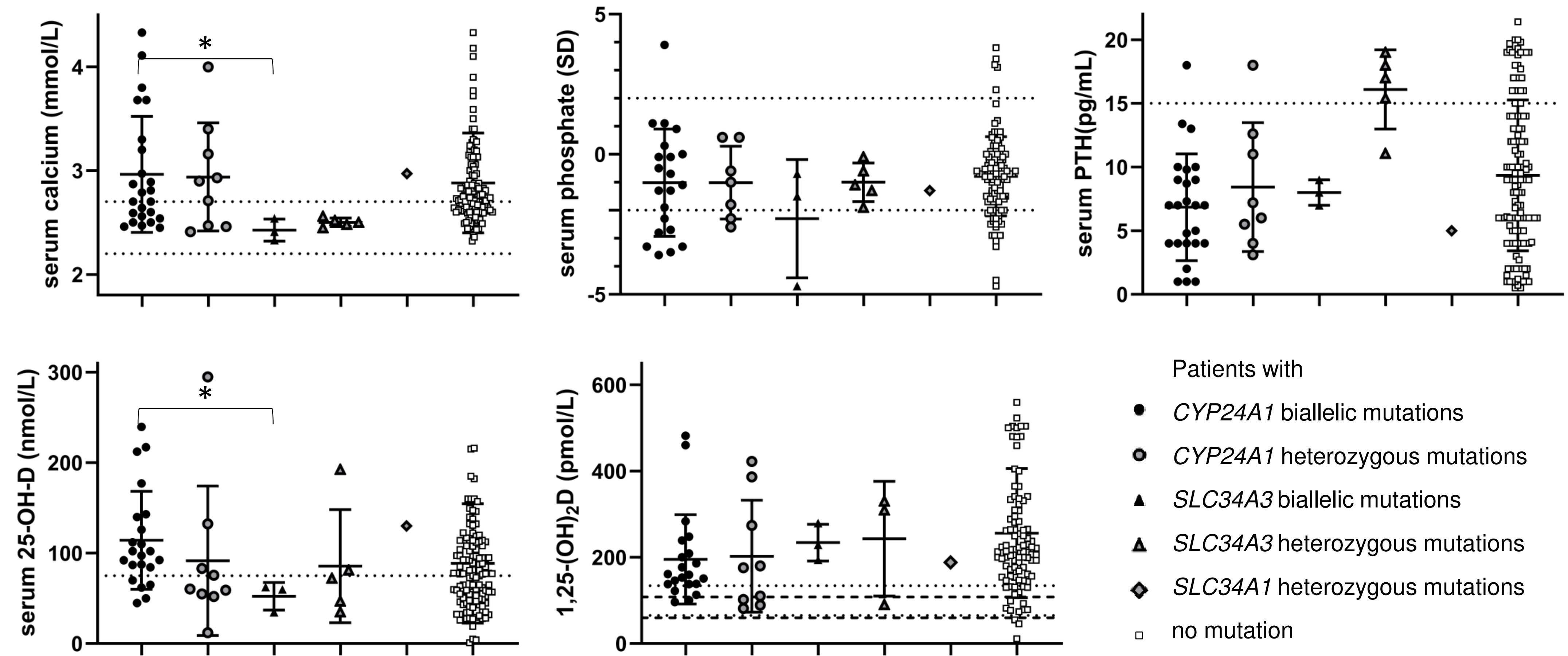
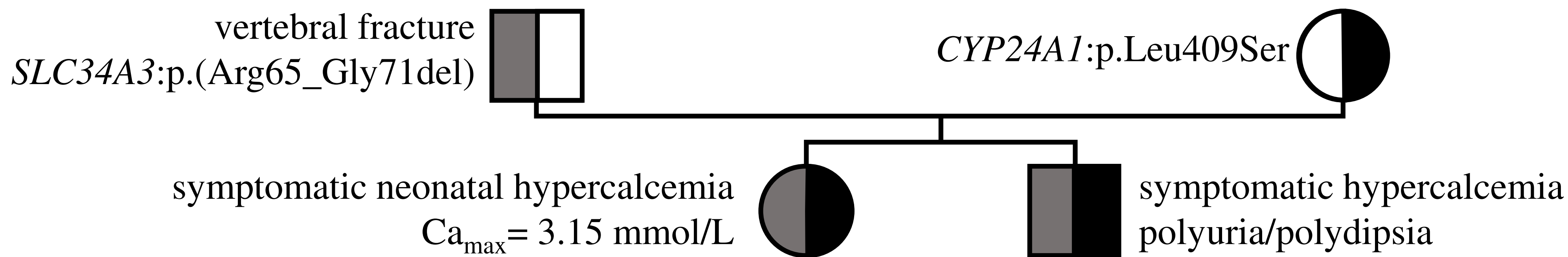


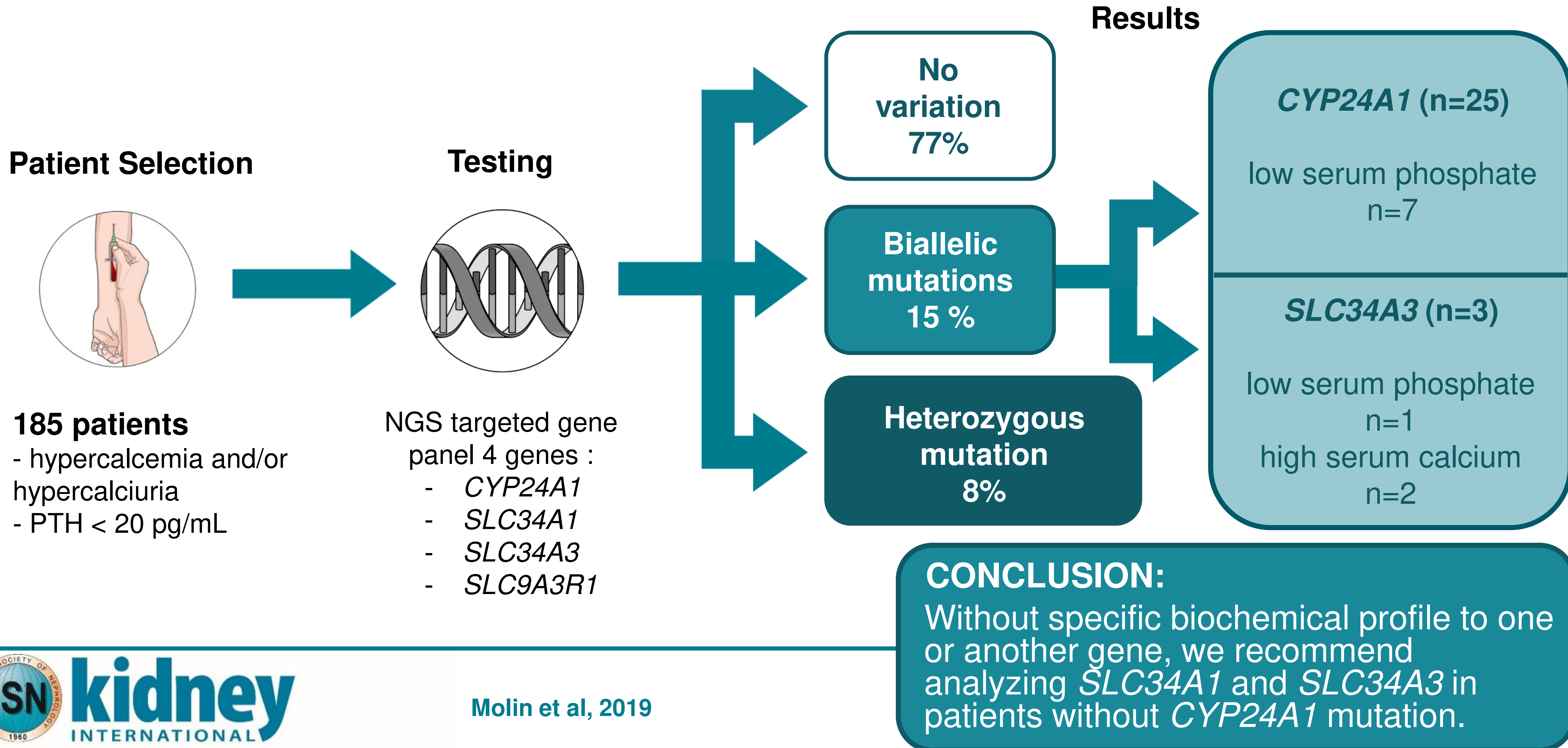
Figure 2



Age	**42 years**	**3 years**	**13 months**	**36 years**
serum calcium (mmol/L) (2.2-2.7)	2.38	2.52	2.51	2.25
serum phosphate (mmol/L)/(SD)	1.18/+0.5	1.53/-1.1	1.53/-1.1	1.1/0
intact PTH (pg/mL) (15-65)	20	9	9	24
25-OH-D (nmol/L) (>50)	74.9	64.9	127.3	74.9
1,25-$(OH)_2$D (pmol/L) adults: 60-108 children: 65-134	105.8	134.6	206.4	139

Ca_{max}: highest serum calcium which had been reported

Overlapping phenotypes associated with *CYP24A1*, *SLC34A1* and *SLC34A3* mutations: a cohort study of patients with hypersensitivity to vitamin D



Données additionnelles présentées lors du 22nd Vitamin D WorkShop (New York, juin 2019)

L'étude de notre cohorte de patients nous a permis de sélectionner 8 patients (**Tableau 6**) porteurs de variations rares du gène *SLC34A1* présentes à l'état hétérozygote composite. La reprise des données biologiques au moment du diagnostic moléculaire et au cours du suivi révèle des fluctuations de la calcémie et de la phosphatémie, tendant vers une normalisation progressive, tandis que la concentration sérique de PTH demeure basse (**Figure 21**).

Chez 4 de ces patients, une hyperéchogénicité rénale sans kyste avait été mise en évidence lors de l'échographie obstétricale du 2ème trimestre de la grossesse (**publication annexe d**).

Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sexe	M	M	M	M	F	M	F	M
Âge (ans*)	10 j	1 m	4,5 m	9 m	1,5	3	6	17
Calcium (mmol/L)	2,99	3,08	2,66	2,64	2,97	2,59	2,83	2,67
Phosphate (mmol/L DS**)	1,60 -2,5	0,99 -3,6	2,0 -0,3	1,39 -2,3	1,7 -1,3	1,44 -1,8	-	1,01 -1,6
PTH (15-65) (pg/mL)	<5	4	8	10,3	<5	12,2	9	23,7
25-OH-D (nmol/L)	-	99,6	57,6	-	130	58,2	42	63,4
1,25-$(OH)_2D$ (15-135) (pmol/L)	283	-	-	-	188	143	230	90,8
calcium/creat. urines (mmol/mmol)	4	-	3	0,64	4,03	-	2,5	-
génotype *SLC34A1*	V91_A97 del I193N	G153V S162Rfs*21	V530M V530M	R495H R495H	V91_A97 del c.1006+1 G>A	I154_V160dup S162R	c.1416+5 G>A T451I	(S483S) P570T
classification ACMG	IV/III	IV/V	III/III	V/V	IV/V	IV/III	III/III	III/III

Tableau 6 : Cohorte de patients avec variations bialléliques de *SLC34A1*

M : mois ; d : jours ** SD calculés d'après les valeurs de Colantonio *et al.* 2012 *Clin Chem* (CALIPER cohort)

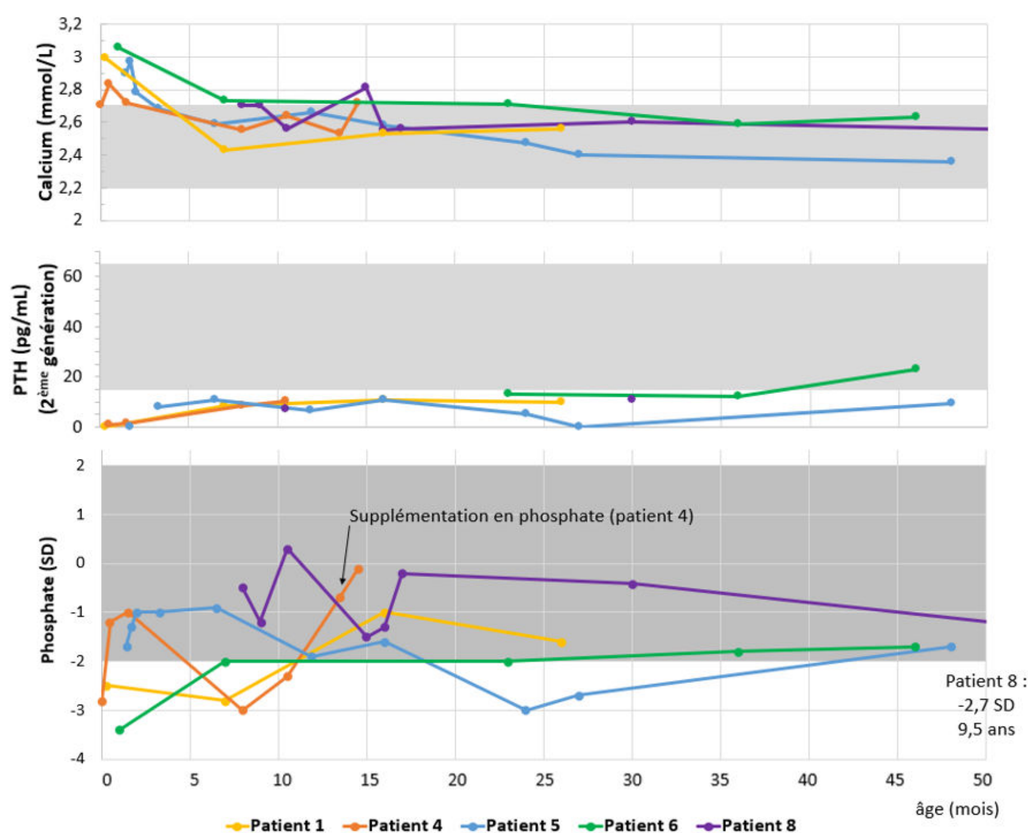


Figure 21 : Evolution de la calcémie, de la phosphatémie et de la PTH chez 5 patients avec variation biallélique de *SLC34A1*

Etude des promoteurs proximaux de *CYP24A1* et *CYP27B1*

L'étude des promoteurs proximaux n'a révélé aucune variation d'intérêt susceptible de modifier les VDRE présents en amont de l'exon 1 du gène *CYP24A1* dans l'ensemble de la cohorte.

dbSNP	Fréquence allélique maximale (GnomAD genome)	Nombre de patients hétérozygotes	Nombre de patients homozygotes
rs778207299	0,1 % population ENF	1	0
rs377383414	1 % population africaine	2	0
rs2762943	98,5 % population africaine	7	0
rs2585427	- (60 % couverture)	35	9
rs187147516	2,3 % population ENF	2	0

Tableau 7 : SNP identifiés lors de l'étude du promoteur de *CYP24A1*

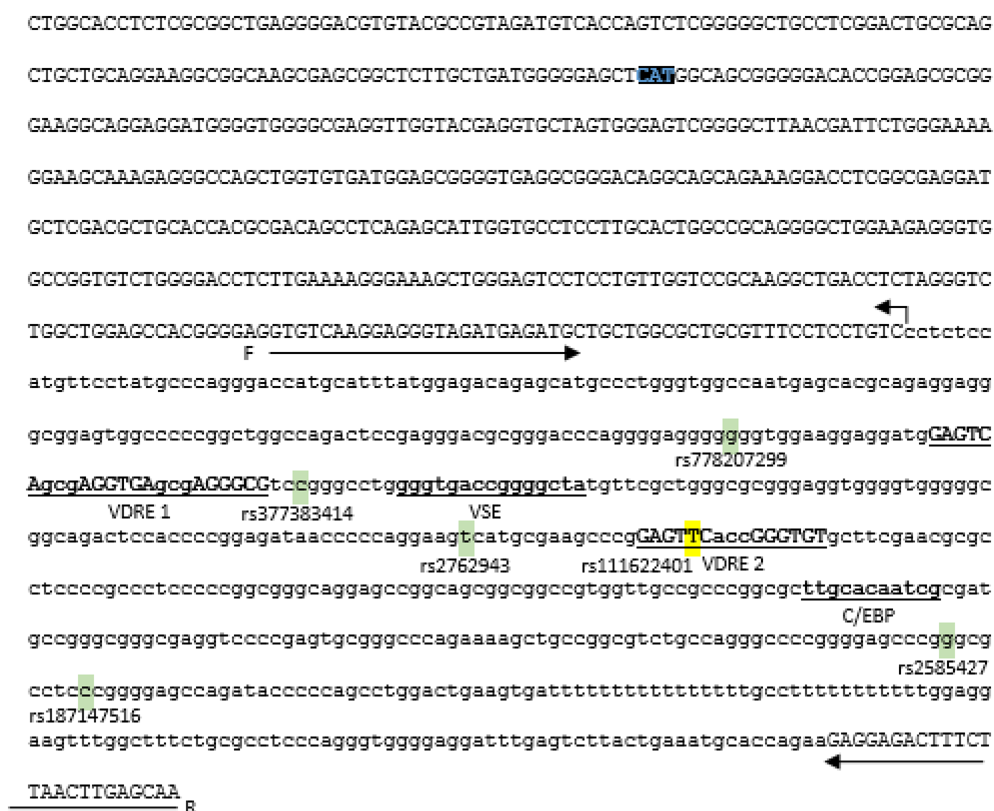


Figure 22 : SNP identifiés lors du séquençage du promoteur de *CYP24A1*

En jaune, SNP identifié par Roff et Wilson. En vert, SNP identifiés dans la cohorte.

Des échantillons de sérum pour étude du ratio 25-OH-D_3/24,25-$(OH)_2D_3$ étaient disponibles pour 3 patients porteurs du SNP rs2585427 à l'état homozygote (**Tableau 8**).

Patient	25-OH-D (nmol/L)	24,25-$(OH)_2D_3$ (nmol/L)	Ratio (N<50)
1	54,5	2,1	26
2	64,3	3,4	18,9
3	69,6	3,8	17,9

Tableau 8 : Rapport 25-OH-D_3/24,25-$(OH)_2D_3$ chez les patients portant le SNP rs2585427 à l'état homozygote

L'étude du promoteur proximal de *CYP27B1* a montré la présence de 5 SNP dont deux rares (fréquence allélique inférieure à 1 %) (**Tableau 9**).

dbSNP	Variation	Fréquence allélique maximale (GnomAD genome)	Nombre de patients hétérozygotes
rs371880778	c.-46_-44del	0,1 % population africaine	1
rs113298639	c.-161A>T	3,6 % population africaine	3
rs148627073	c.-775C>T	0,5 % population ENF	1
rs11172327	c.-836C>T	5,2 % population africaine	2
rs142121467	c.-895A>T	1 % population africaine	1

Tableau 9 : SNP identifiés lors de l'étude du promoteur proximal de *CYP27B1*

A noter la présence d'un sujet hétérozygote pour les variations rs113298639 et rs11172327.

ENF : European non Finnish.

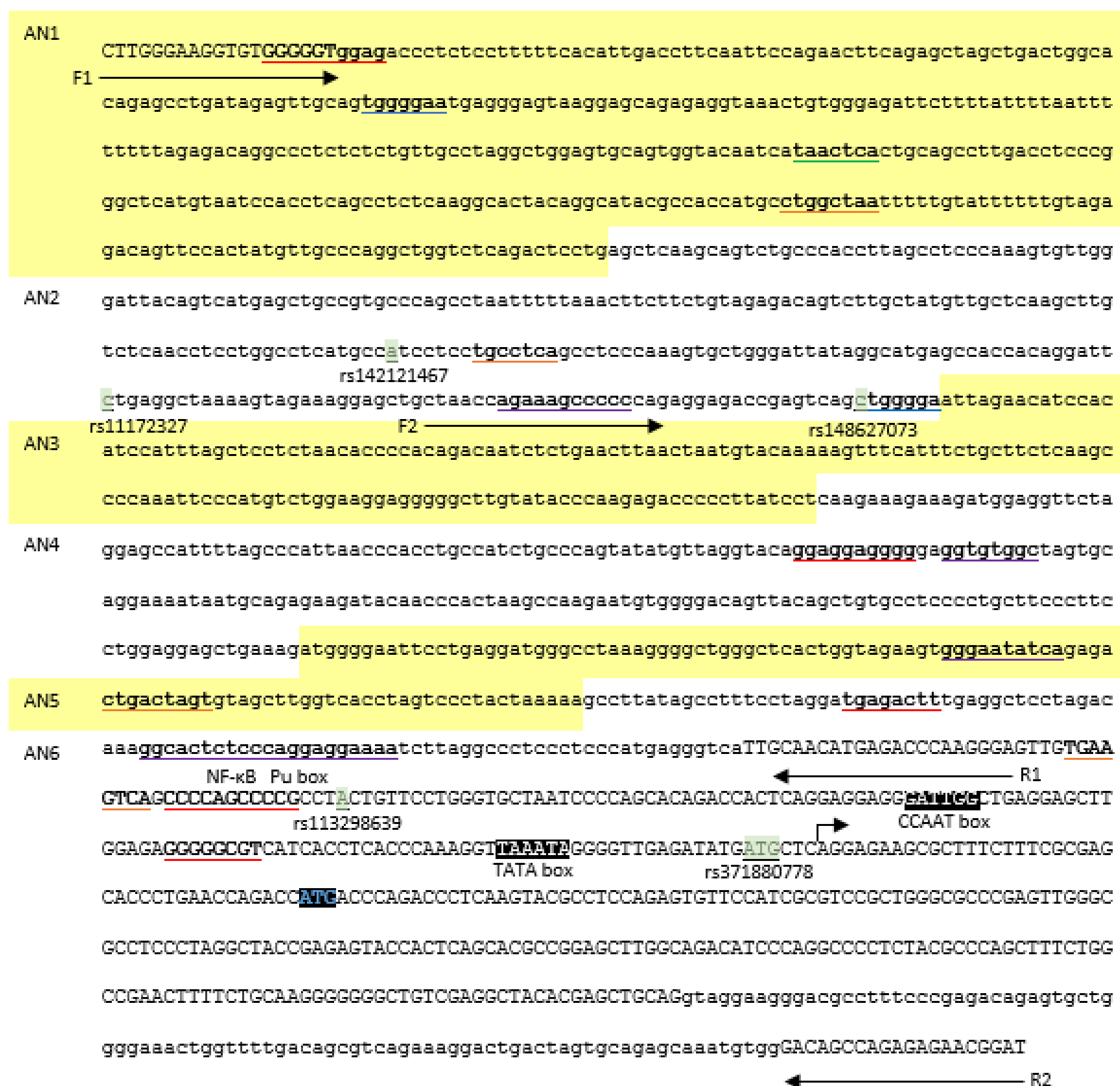


Figure 23 : SNP identifiés lors du séquençage du promoteur de *CYP27B1*
Sites de fixation aux facteurs de transcription déterminés par analyse bioinformatique (rouge : Sp1, bleu : AP-2, vert : AP-1, orange : cAMP Responsive Element, violet : NF-κB). En vert, SNP identifiés dans la cohorte.

Il n'a pas été identifié d'anomalie récurrente ni de grand réarrangement de cette région par la technique utilisée.

Données additionnelles non publiées : cas clinique de M. V.

L'exploration d'un patient de 30 ans en raison d'une hypercalcémie chronique évoluant dans un contexte de syndrome polyuropolydipsique évoluant depuis l'enfance a révélé la présence

d’une mutation hétérozygote c.1186C>T /p.Arg396Trp à l’état hétérozygote et d’un rapport 25-OH-D / 24,25-$(OH)_2D$ élevé en faveur d’un déficit en vitamine D 24-hydroxylase.
La recherche de grand réarrangement (délétion ou duplication intragénique) n’a identifié aucune anomalie.
Le séquençage du promoteur de *CYP24A1* a révélé la présence d’une variation de séquence rare rs2585427 localisée 460 pb en amont du site d’initiation de la transcription, en dehors de tout site de liaison à un facteur de transcription actuellement identifié. Le ratio 25-OH-D / 24,25-$(OH)_2D$ est normal chez les sujets homozygotes pour ce SNP (**Tableau 8**), ce qui n’est pas en faveur d’un défaut d’expression de *CYP24A1* imputable à cette variation.
Le séquençage du promoteur de *CYP27B1* n’a révélé aucune variation de séquence rare.

DISCUSSION GENERALE

L'objectif de ce travail de thèse était d'identifier des mécanismes génétiques de dérégulation du métabolisme de la vitamine D en étudiant une cohorte de patients présentant des profils biochimiques particuliers potentiellement en lien avec une maladie rare. Notre réflexion s'est organisée autour de deux grands axes : dans un premier temps, rechercher des anomalies de gènes codant les hydroxylases directement ou potentiellement impliquées dans le métabolisme de la vitamine D, dans un second temps, rechercher des anomalies génétiques responsables d'une dérégulation du métabolisme de la vitamine D.

Vitamine D 1α-hydroxylase (*CYP27B1*)

Nous ne souhaitions pas inclure dans ce travail les patients présentant des mutations perte de fonction de *CYP27B1* (vitamine D 1α-hydroxylase) responsables du VDDR1A dont le phénotype et la prise en charge sont mieux décrits[76,222–227]. Néanmoins, l'utilisation d'échantillons de sérum de patients malades, avec et sans traitement, nous a permis d'étudier le rapport 25-OH-D_3/24, 25-$(OH)_2D_3$ et de confirmer chez l'Homme qu'une concentration sérique normale de 1,25-$(OH)_2D$ est nécessaire à l'expression rénale de *CYP24A1*. En effet, ce rapport s'élève chez le patient VDDR1A sans traitement, malgré une 25-OH-D normale, et se normalise chez les patients traités[261], ce qui reproduit les données précédemment publiées dans le modèle murin de cette maladie[262].

Inversement, les causes de surexpression ou d'augmentation de l'activité de la vitamine D 1α-hydroxylase sont moins bien décrites, en particulier sur le plan génétique. L'hypothèse de variations gain de fonction de *CYP27B1*, augmentant l'activité de l'enzyme ou son affinité pour le substrat, avait été avancée pour expliquer le phénotype d'hypersensibilité à la vitamine D. L'analyse de *CYP27B1* est incluse dans le panel « Maladie rare du métabolisme de la vitamine D » du laboratoire dont l'utilisation a permis de rechercher des variations chez des patients présentant des taux hauts et inadaptés de 1,25-$(OH)_2D$ malgré une PTH basse, soit un profil biochimique compatible avec un gain de fonction de la vitamine D 1α-hydroxylase.

Cette stratégie n'a permis l'identification que d'une seule variation faux-sens NM_000785.4:c.496G>C (p.Val166Leu) (rs8176344) à l'état hétérozygote chez un patient présentant également une variation faux-sens hétérozygote du gène *SLC34A3*. Cette variation

de *CYP27B1* a été précédemment associée à un gain de fonction[260]. Néanmoins, les données de fréquence la concernant (base de données GnomAD exome) montrent une fréquence allélique élevée dans la population asiatique (6,4%) plutôt en faveur d'un polymorphisme. Bien que ce résultat puisse également suggérer une dérégulation par digénisme, l'existence d'un élément biologique en faveur d'une cause extrarénale d'hypercalcitriolémie (dosage de l'enzyme de conversion) n'est pas en faveur d'une dérégulation du métabolisme de la vitamine D d'origine génétique chez ce patient. L'hypothèse de variations gain de fonction de *CYP27B1* n'a donc pas pu être vérifiée dans notre cohorte de patients, et n'a fait l'objet d'aucune publication additionnelle depuis le début de ce travail. Notre attention s'est donc focalisée sur les 25- et la 24-hydroxylases.

Vitamine D 25-hydroxylases (*CYP2R1* et *CYP3A4*)

Dans un premier temps, nous souhaitions rechercher des variations gain de fonction d'une 25-hydroxylase susceptible d'expliquer une concentration sérique plus élevée de 25-OH-D (substrat), et donc de 1,25-$(OH)_2$D (produit), chez des patients recevant une supplémentation à dose habituelle en vitamine D (population pédiatrique essentiellement), voire en l'absence de supplémentation en vitamine D (population adulte essentiellement). Notre étude s'est ainsi attachée à rechercher des variations potentiellement pathogènes de *CYP2R1*, gène supposé coder la vitamine D 25-hydroxylase principalement active sur le plan physiologique, et de *CYP3A4*, gène codant une enzyme vitamine D 25-hydroxylase accessoire principalement impliquée dans le métabolisme de xénobiotiques et cytochrome P450 le plus abondant dans le foie. D'autres enzymes à activité vitamine D 25-hydroxylase sont également décrites chez l'Homme, mais n'ont pas été considérées dans notre étude : le gène *CYP27A1* codant une enzyme capable de réaliser la 25-hydroxylation de la vitamine D_3 et le gène *CYP2J2* codant une enzyme à activité vitamine D 25-hydroxylase capable de réaliser la 25-hydroxylation de la vitamine D_2. Les mutations perte de fonction de *CYP27A1* sont associées à la xantomatose cérébrotendineuse (OMIM 212700), maladie autosomique récessive de la synthèse des sels biliaires à expression syndromique (cholestase, cataracte précoce, atteinte neurologique). Il n'a donc pas été étudié, le phénotype associé aux variations de ce gène semblant trop sévère. Concernant *CYP2J2*, la consultation des bases de données de variations génétiques montre une probabilité nulle d'intolérance à la perte de fonction (pLI=0), peu en faveur d'une implication de ce gène en pathologie humaine.

Il n'a pas été mis en évidence de variation pathogène de *CYP2R1* chez des sujets présentant un phénotype HVD, quelle que soit la concentration en 25-OH-D. En revanche, notre travail a permis l'identification de mutations perte de fonction de *CYP2R1* dans deux familles avec VDDR1B (**publication B**). Notre étude renforce ainsi le rôle de CYP2R1 comme enzyme physiologiquement impliquée dans la 25-hydroxylation de la vitamine D tout en soulignant le rôle des 25-hydroxylases accessoires (efficacité relative du traitement par 1α-OH-D_3). Ces dernières, recrutées en cas de supplémentation à dose supraphysiologique, demeurent inaptes à garantir une homéostasie phosphocalcique en cas de déficit complet de CYP2R1.

Nos observations remettent également en question le schéma classique désignant la 25-OH-D comme une simple forme de stockage et la 1,25-$(OH)_2D$ comme la seule forme active. En effet, l'observation d'un phénotype pathologique chez des patients présentant des concentrations sériques normales de 1,25-$(OH)_2D$ mais indétectables de 25-OH-D d'une part, d'une amélioration biochimique incomplète après traitement par 1α-OH-D_3 avec augmentation majeure de la concentration en 1,25-$(OH)_2D$ (ALP et PTH demeurant augmentées) d'autre part, suggère des effets biologiques propre à la 25-OH-D. Il est donc vraisemblable que la 25-OH-D possède une activité métabolique malgré une affinité 500 fois plus faible pour le récepteur de la vitamine D, potentiellement compensée par une concentration 1000 fois plus grande. Un rôle métabolique passant à la synthèse de métabolites dont les effets sont encore aujourd'hui mal connus (comme la 24,25-$(OH)_2D$ par exemple) pourrait également être envisagé. La question de l'impact des mutations perte de fonction hétérozygotes de *CYP2R1* sur le métabolisme phosphocalcique reste néanmoins en suspens du fait du faible nombre de sujets hétérozygotes identifiés dans notre cohorte. Il pourrait s'agir d'un facteur de risque génétique de carence en vitamine D.

Nous n'avons pas identifié de mutation dans le gène *CYP3A4*, quel que soit le phénotype. En revanche, en 2018, Roizen *et al.* [263] identifièrent par exome une mutation gain de fonction hétérozygote *de novo* NM_017460.5 :c.902T>C (p.Ile301Thr) de ce gène chez deux patientes non apparentées présentant un phénotype de rachitisme avec 25-OH-D indétectable évocateur de VDDR1B sans mutation de *CYP2R1*. Il décrivirent néanmoins une concentration en 1,25-$(OH)_2D$ basse ou indétectable, qui diffère significativement du phénotype de nos patients avec VDDR1B à 1,25-$(OH)_2D$ normale ou haute. Les études de fonctionnalité révélèrent que cette mutation confère à l'enzyme CYP3A4 une activité catalytique accrue (élévation du rapport 4β,25-$(OH)_2D$/25-OH-D ; la 4β,25-$(OH)_2D$ étant le produit de dégradation de la 25-OH-D par CYP3A4), supérieure à celle de l'enzyme sauvage mais aussi à celle de CYP24A1. Ils

nommèrent cette nouvelle maladie autosomique dominante rachitisme pseudocarentiel de type 3 (VDDR3, *vitamin D–dependent rickets type 3*).

Ainsi, bien que la carence en vitamine D reste le diagnostic le plus rapidement évoqué en cas de rachitisme à 25-OH-D basse, la survenue d'un tableau clinique et biologique compatible chez un enfant bénéficiant d'une supplémentation adaptée, sans défaut d'observance et sans malabsorption, peut faire évoquer deux maladies génétiques, l'une autosomique récessive (type 1B), l'autre autosomique dominante (type 3). Outre le conseil génétique, leur caractérisation sur le plan moléculaire garantit une prise en charge personnalisée et adaptée au mécanisme physiopathologique, basée respectivement sur un traitement par 25-OH-D (type 1B) ou sur de forte dose de vitamine D_3 ou de calcitriol (type 3).

Vitamine D 24-hydroxylase (*CYP24A1*)

Notre travail (**publication A**) porta ensuite sur l'étape de dégradation de la 1,25-$(OH)_2$D, c'est-à-dire sur le gène *CYP24A1* codant la vitamine D 24-hydroxylase. Des mutations perte de fonction de ce gène furent associées à l'hypercalcémie infantile idiopathique (IIH) en 2011 par Schlingmann *et al.*[115]. Plusieurs familles furent initialement publiées, compatibles avec une hérédité autosomique récessive. L'hypothèse d'une forme autosomique dominante de déficit en vitamine D 24-hydroxylase fut néanmoins proposée par Tebben *et al.*[256].

Nos travaux permirent de préciser l'hérédité de cette maladie grâce à l'utilisation d'un nouveau biomarqueur, le ratio 25-OH-D_3/24, 25-$(OH)_2D_3$, qui constitue à la fois un outil de diagnostic *in vivo* du déficit en vitamine D 24-hydroxylase, mais également un outil d'aide à l'interprétation des variations géniques identifiées par séquençage.

Premièrement, l'élévation du rapport 25-OH-D_3/24, 25-$(OH)_2D_3$ (substrat/produit) n'est observée que chez les patients avec mutations bialléliques ; le ratio reste normal chez les sujets hétérozygotes, et en particulier, chez les sujets apparentés hétérozygotes obligatoires asymptomatiques (parents des enfants atteints principalement). Néanmoins, nous observons une concentration sérique en 1,25-$(OH)_2$D élevée chez ces sujets hétérozygotes ; cette observation suggère un impact de ces mutations sur le métabolisme de la vitamine D, en cohérence avec la fonction première de dégradation de la 1,25-$(OH)_2$D de cette enzyme et peut-être, d'un effet de dosage génique. S'il n'est pas possible actuellement d'exclure toute conséquence clinique de ces variations hétérozygotes de *CYP24A1* sur le phénotype des patients (facteur de risque de

lithiase rénale ou d'ostéoporose notamment), le phénotype des patients hétérozygotes apparait donc bien différent de celui des malades avec mutations bialléliques.

Deuxièmement, le ratio constitue une aide précieuse à l'interprétation des variations identifiées par séquençage, faux-sens en particulier. Ainsi, l'observation d'un ratio normal chez des patients porteurs à l'état hétérozygote composite des variations p.Met374Thr et p.Arg157Trp prédites pathogènes par les analyses bioinformatiques permit de considérer ces variations comme bénignes. Notre publication de 2018 (**publication C**) sur une délétion partielle de *CYP24A1* appuie également l'apport de l'analyse familiale et de la recherche de grands réarrangements de ce gène. Cet article souligne l'existence de mutations potentiellement non détectées par l'ensemble des techniques de séquençage habituellement employées (Sanger en particulier), mais indirectement identifiable grâce au ratio. Ainsi, les résultats obtenus lors de l'exploration de M. V. démontre l'intérêt de l'utilisation combinée de l'exploration génétique et du rapport 25-OH-D_3/24, 25-$(OH)_2D_3$ permettant de distinguer les hétérozygotes vrais (à ratio normal) des patients dont la deuxième mutation n'a pas été détectée (à ratio élevé). Chez ce patient, le ratio élevé confirme un déficit enzymatique complet malgré l'identification d'une seule variation hétérozygote dans la séquence codante de *CYP24A1*. Un second évènement reste donc à préciser, potentiellement dans la partie non codante du gène ou dans une séquence régulatrice.

Physiologiquement, la synthèse d'une vitamine D 24-hydroxylase fonctionnelle est liée à l'expression d'un gène *CYP24A1* fonctionnel en présence d'une concentration suffisante en 1,25-$(OH)_2D$ (quantité de substrat suffisante et gène *CYP27B1* fonctionnel) et d'un récepteur VDR fonctionnel capable de se fixer au niveau de régions régulatrices. Un défaut d'expression d'un gène *CYP24A1* normal pourrait être dû à un défaut d'un facteur activant sa transcription (mutations perte de fonction du VDR par exemple[264]) ou de son site de fixation à l'ADN (VDRE nécessaire à la fixation du complexe VDR-RXR par exemple). La variation rs111622401 précédemment publiée par Roff et Wilson[133] au niveau d'un VDRE du promoteur proximal du gène illustre cette hypothèse, sa présence diminuant l'expression du gène induite par la 1,25-$(OH)_2D$ dans le modèle cellulaire utilisé par ces auteurs.

Notre objectif a donc été de rechercher de telles variations susceptibles de modifier la liaison récepteur-promoteur chez M. V. et chez l'ensemble des patients de notre cohorte. Aucune variation de séquence affectant la séquence des VDRE n'a été identifiée, bien que la technique utilisée ne permette pas d'exclure la présence d'un grand réarrangement (délétion de la région promotrice toutefois exclue chez les patients présentant une variation hétérozygote dans le

promoteur dont on a *de facto* étudié les deux allèles). La localisation chez l'Homme et l'étude d'autres régions régulatrices situées à distance, comme celles identifiées par Meyer *et al.*[134] chez la souris, parait donc souhaitable.

Facteurs régulant la vitamine D 1α-hydroxylase

Par la suite, selon une stratégie gène candidat, plusieurs autres gènes impliqués dans le métabolisme de la vitamine D ou le contrôle de l'expression de la 1α-hydroxylase furent inclus dans notre panel d'analyse. Parmi eux, les gènes codant la protéine de transport de la vitamine D, *GC*, et ceux codant ses récepteurs, *VDR* codant le récepteur à la vitamine D et *PDIA3* supposé coder le récepteur impliqué dans la voie non génomique. Nous souhaitions rechercher des variations associées à une augmentation de la biodisponibilité de la vitamine D (gain de fonction de la protéine de transport) ou à une activation constitutive de ses récepteurs. Aucune variation d'intérêt n'a été identifiée dans ces gènes dans notre cohorte.

Trois gènes impliqués dans le contrôle de la réabsorption tubulaire de phosphate (*SLC34A1*, *SLC34A3* et *SLC9A3R1* codant respectivement les cotransporteurs rénaux sodium-phosphate NPT2a et c et la protéine adaptatrice NHERF1) furent également étudiés chez nos patients. Notre hypothèse était qu'une anomalie génétique associée à une diminution de la concentration sérique de FGF23 pourrait conduire à une dérégulation de la 1α-hydroxylase (concept de levée d'inhibition). En effet, les patients porteurs de rachitisme hypophosphatémique à concentration sérique de FGF23 haute inadaptée présentent des concentrations sériques de 1,25-$(OH)_2$D basses (par exemple, dans l'hypophosphatémie liée à l'X par mutation perte de fonction du gène *PHEX*[265]). Inversement, les patients porteurs de calcinose tumorale hyperphosphatémique (mutation perte de fonction de *FGF23* par exemple[266]) présentent des concentrations sériques de 1,25-$(OH)_2$D élevées. Cette hypothèse était également étayée par les études portant sur les modèles murins Slc34a1$^{-/-}$[267] et Slc34a3$^{-/-}$ et par les phénotypes des patients humains avec mutation de *SLC34A1*[268] ou *SLC34A3*[269] caractérisés par une concentration sérique en 1,25-$(OH)_2$D élevée.

Notre travail (**publication D**) nous permit d'identifier des variations rares des gènes *SLC34A1* et *SLC34A3*, la plupart considérée comme de signification indéterminée selon les critères du Collège américain de Génétique médical, une minorité ayant été précédemment publiée. Il souligne le chevauchement phénotypique entre déficit en vitamine D 24-hydroxylase et défaut de la réabsorption tubulaire de phosphate, explicable par la régulation réciproque de la 1,25-

$(OH)_2D$ par le FGF23 et du FGF23 par la 1,25-$(OH)_2D$, et par leur position centrale dans la régulation du métabolisme phosphocalcique.

Ce travail illustre également l'importance des facteurs environnementaux dans les maladies d'origine génétique. En effet, dans le déficit en vitamine D 24-hydroxylase, nous soulignons le rôle majeur de la concentration sérique basse de PTH comme marqueur de cette maladie chez des patients ne présentant pas d'hypercalcémie. C'est l'environnement (apport en vitamine D ou ensoleillement) qui conduit à un déséquilibre métabolique franc avec hypercalcémie et PTH indétectable chez ces patients. Nous évoquons le même type de mécanisme physiopathologique chez les patients présentant des variations de *SLC34A1* et *SLC34A3*, dont la phosphatémie peut fluctuer au cours du temps mais dont la PTH reste basse et la 1,25-$(OH)_2D$ haute (données présentées lors du *22nd Vitamin D workshop*). Des études de corrélation génotype-phénotype seront nécessaires pour déterminer s'il s'agit de fluctuations liées au caractère complet ou incomplet de la perte de fonction associée aux variations géniques de *SLC31A1* et *SLC34A3*.

Enfin, l'observation d'une famille particulière où ségrégent une mutation *CYP24A1* et une variation rare de signification indéterminée de *SLC34A3* suggère l'hypothèse d'une dérégulation de la 1α-hydroxylase par mécanisme digénique, hypothèse préalablement proposée par Tabibzadeh *et al.* [270]. Le phénotype de ces patients résulterait de l'effet cumulé de deux variations hétérozygotes associées chacune à une élévation du calcitriol sérique (élévation du calcitriol sérique observée chez les hétérozygotes *CYP24A1* dans notre cohorte et chez les hétérozygotes *SLC34A3* dans l'étude publiée par Dasgupta *et al.*). Ce mécanisme physiopathologique pourrait expliquer des proportions variables de sujets symptomatiques parmi les patients hétérozygotes pour des mutations de *SLC34A1*[257], *SLC34A3*[269] ou *CYP24A1*[256].

Plus largement, ce type d'hypothèse physiopathologique mêlant déséquilibre du métabolisme du phosphate et de la vitamine D pourrait expliquer une partie des hypersensibilités à la vitamine D néonatales transitoires identifiées chez une majorité d'enfants de notre cohorte. Ce phénotype résulterait de la conjonction d'une faiblesse relative des apports en phosphate (favorisée par la prématurité et/ou un allaitement maternel exclusif) activant l'expression de la vitamine D 1α-hydroxylase (baisse du FGF23) et des apports relativement excessifs en vitamine D (supplémentation recommandée dès les premiers jours de vie) apportant en trop grande quantité un substrat à une machinerie enzymatique poussée à plein régime.

Il n'existe pas à notre connaissance de donnée épidémiologique sur l'hypersensibilité à la vitamine D et l'hypercalcémie néonatale. Néanmoins, l'absence de complication chez une majorité d'enfants bénéficiant d'une supplémentation en vitamine D suggère l'existence de facteurs de risques potentiellement génétiques chez nos patients, comprenant éventuellement des mutations hétérozygotes des gènes susmentionnés. L'absence de variation dans la séquence codante de ces gènes chez une majorité d'enfant pourraient être en faveur de variations génétiques à risque localisées en dehors des séquences codantes et pouvant influer sur l'expression des gènes. L'expression de *CYP27B1* et *CYP24A1* étant finement régulée de façon opposée et synchrone (ce qui active l'expression de l'un inhibe celle de l'autre et réciproquement), la présence de variation(s) dans les séquences promotrices représente une hypothèse de travail intéressante.

L'analyse du promoteur proximal de *CYP27B1* a été réalisée suivant la même méthodologie de séquençage Sanger que celle utilisée pour le promoteur de *CYP24A1*, bien qu'aucune variation susceptible de modifier l'expression du gène n'ait à ce jour été publiée. Seule les données bibliographiques et bioinformatiques purent être utilisées pour interpréter les variations de séquence identifiées chez nos patients[130]. Il n'a pas été identifié de variation jugée significative dans les régions du promoteur précédemment publiées comme activatrices de l'expression du gène. Seule une variation hétérozygote impactant un site de fixation à un facteur de transcription non spécifique de type AP-2 a été mise en évidence. En particulier, aucune variation dans un site de type *cAMP Response Element* (élément de réponse à l'AMP cyclique, second messager émis suite à l'activation de la voie en aval du récepteur à la PTH) n'a été détectée. Là encore, la technique employée ne permet pas d'exclure un réarrangement de grande taille chez les sujets ne présentant aucun polymorphisme hétérozygote. La séquence étudiée ne correspond qu'au promoteur proximal et on ne peut exclure que des anomalies de régulateurs distaux puissent être impliquées.

En effet, lors de la rédaction de ce manuscrit, un nouvel article portant sur la caractérisation chez la souris d'éléments régulateurs de l'expression de Cyp27b1 localisés à distance du gène, dans les introns des gènes Mettl21b et Mettl1 (pour *Methyltransferase-like 21b* et *1*) parut dans *Journal of Biological Chemistry*[271]. Ce travail suggère une organisation similaire des loci murins et humains et l'existence potentielle d'éléments régulateurs dans les introns 2 et 3 des gènes *METTL1* et *METTL21B* (aussi nommé *EEF1AKMT3* pour *EEF1A Lysine Methyltransferase 3*). D'après les études fonctionnelles réalisées chez la souris, et en supposant l'existence de similitudes fonctionnelles chez l'Homme, des anomalies des éléments

régulateurs contenus dans ces introns pourraient être impliquées en pathologie humaine. Ainsi, des variations introniques de *EEF1AKMT3* pourraient être responsables de défaut d'inhibition de la 1α-hydroxylase par défaut de réponse au FGF23 (phénotype HVD avec hypophosphatémie et FGF23 élevé). Des anomalies du régulateur inclus dans *METTL1* pourraient être responsables d'une résistance à la PTH (phénotype évocateur de pseudohypoparathyroïdie ou de VDDR1A) ou d'un défaut de rétrocontrôle de la 1,25-$(OH)_2D$. Ces éléments représentent de nouvelles pistes d'exploration.

CONCLUSION GENERALE ET PERSPECTIVES

En conclusion, notre travail illustre l'intérêt d'une stratégie d'étude combinant analyse biochimique par LC-MS/MS et analyse génétique pour l'étude des maladies rares du métabolisme de la vitamine D. Il est vraisemblable que l'utilisation commune de ces deux méthodologies permettra d'identifier de nouveaux métabolites d'intérêt (comme la 24,25-$(OH)_2D$ produite par la 24-hydroxylase CYP24A1 sauvage ou la 4β,25-$(OH)_2D$ produite par l'enzyme CYP3A4 mutée) utiles à l'interprétation des données génétiques et à la compréhension du métabolisme et des nombreux effets physiologiques de la vitamine D.
Ce travail souligne aussi l'intérêt de l'étude de la régulation de l'expression des gènes impliqués dans le métabolisme de la vitamine D, étude des régions régulatrices de ces gènes et de leur régulateurs *trans*, mais aussi étude des gènes impliqués dans d'autres mécanismes physiologiques pouvant secondairement impacter le métabolisme de la vitamine D (homéostasie du phosphate).

Plusieurs voies s'offrent maintenant à nous pour la poursuite de ce travail.

Premièrement, l'identification de variations génétiques potentiellement pathogènes dans des gènes indirectement liés au métabolisme de la vitamine D (transporteurs de phosphate) suggère qu'il nous faut toujours considérer l'hypothèse d'un déséquilibre plus global impactant secondairement le métabolisme de la vitamine D (maladie rénale ou hépatique, maladie du métabolisme du fer impactant FGF23 par exemple). L'utilisation de stratégies d'étude pangénomique type séquençage d'exome ou de génome (*whole exome/genome sequencing*) et du phénotypage inversé (*reverse phenotyping*) devrait permettre d'élargir le spectre phénotypique de maladies déjà décrites mais actuellement non connues pour impacter le métabolisme de la vitamine D, voire d'identifier de nouvelles maladies génétiques. Le développement d'étude de fonctionnalité s'imposera dans ce contexte, qu'il s'agisse de modèles cellulaires ou animaux, voire de l'utilisation de nouveaux biomarqueurs de fonctionnalité *in vivo.* A court terme, il nous faudra ainsi pouvoir accéder à un modèle de fonctionnalité pour évaluer la pathogénicité des variations identifiées dans les gènes *SLC34A1* et *SLC34A3*, qu'il s'agisse d'un développement ou de collaboration.

Deuxièmement, les travaux récents réalisés sur le modèle murin questionnent le rôle des domaines topologiquement associés (TAD) et d'éléments régulateurs distaux dans la physiopathologie du métabolisme de la vitamine D. Il nous faut maintenant appliquer ces connaissances à l'Homme afin de préciser les bornes de ces domaines de régulation, de localiser précisément les éléments régulateurs, d'en identifier les partenaires (quelles séquences de fixation et quels facteurs de transcription) et de caractériser leur fonctionnement à l'échelle de la chromatine. Nous combinerons pour cela les données issues de séquençage du génome ou de séquençage ciblé de patients d'intérêt afin d'identifier d'éventuelles anomalies génétiques à l'origine de défaut d'expression des gènes impliqués dans le métabolisme de la vitamine D à l'étude de prélèvement humains par ChIP-chip, ChIP-seq et analyse de conformation de la chromatine (3C pour *Chromosome Conformation Capture* et techniques dérivées). L'étude de prélèvements fœtaux devrait nous permettre d'étudier la mise en place de ces mécanismes de régulation au cours du développement afin de mieux comprendre la survenue des hypersensibilités à la vitamine D néonatale transitoire.

Enfin, si la vitamine D 25-hydroxylase (*CYP2R1*) ne semble pas aussi finement régulée que les autres enzymes clés du métabolisme de la vitamine D, les mécanismes moléculaires impliqués dans son expression (promoteur proximal, régulateurs distaux, TAD) ne sont actuellement pas connus. Les étudier pourrait néanmoins amener des hypothèses intéressantes pour la prise en charge de certaines maladies fréquentes potentiellement influencées par la vitamine D (ostéoporose, diabète, sclérose en plaques, …).

Ces connaissances de la physiopathologie constituent le socle d'une prise en charge adaptée à la maladie et au patient, dans un concept de médecine personnalisée, pour les maladies communes comme pour les maladies rares. Il s'agit probablement en partie de l'avenir de la génétique, en tant que science et discipline médicale, dont le but n'est plus seulement de décrire et d'étiqueter les phénotypes, mais d'en comprendre les mécanismes de survenue afin d'y proposer des contrepoids efficaces.

Ce travail a permis de disséquer les mécanismes physiopathologiques du déficit en vitamine D 24-hydroxylase, de préciser le phénotype de cette maladie afin de limiter l'errance diagnostique des patients et de faciliter l'accès au diagnostic grâce au développement en routine de l'analyse du ratio 25-OH-D/24,25-$(OH)_2$D au CHU de Caen.

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Annales d'Endocrinologie 77 (2016) 615–619

Hyperparathyroidism complicating *CYP 24A1* mutations

Hyperparathyroïdie compliquant une hétérozygotie composite du gène CYP24A1

Abstract

CYP24A1 gene mutations induce infantile hypercalcemia, with high 1,25$(OH)_2$D contrasting with low PTH levels. The adult phenotype is not well known. Two unrelated adult patients were referred for nephrolithiasis, hypertension, hypercalcemia, hypercalciuria, normal 25-OHD levels, and inappropriate PTH levels (22 to 92 pg/mL; N: 15–68) suggesting primary hyperparathyroidism, leading to surgery. Hypercalciuria improved despite persistent hypercalcemia, treated with cinacalcet. The ratio 25-OHD_3/24-25-(OH)2D_3 > 100 (N < 25) suggested the diagnosis of *CYP24A1* mutations which were confirmed through Sanger sequencing. In conclusion, the adult phenotype associated with *CYP24A1* mutations can evolve over time from hypercalcemia with suppressed PTH towards hyperparathyroidism with moderately increased PTH level, adenoma and/or slightly increased parathyroid glands. Surgery decreased calciuria and improved kidney function. Cinacalcet was partially effective on hypercalcemia since PTH was inappropriate. This novel phenotype, a phenocopy of hyperparathyroidism, might evolve in few cases towards hyperparathyroidism despite random association of the 2 diseases cannot be excluded.
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Résumé

Les mutations du gène *CYP24A1* entraînent une hypercalcémie infantile, caractérisée par des concentrations élevées de 1,25$(OH)_2$D contrastant avec des valeurs basses de PTH. Le phénotype adulte est mal connu. Nous rapportons deux cas adultes souffrant de néphrolithiases, tendinites et hypertension, adressés pour hypercalcémie, hypercalciurie, valeurs normales de 25-OHD mais inappropriées de PTH (22 à 92 pg/mL ; N : 15–68), suggérant une hyperparathyroïdie primaire qui est opérée. L'hypercalciurie s'améliore en dépit d'une hypercalcémie persistante, traitée par cinacalcet. Le rapport 25-OHD_3/24-25-(OH)2D_3 élevé (> 100 [N < 25]) orientait vers une mutation *CYP24A1*, qui fut ensuite confirmée par technique Sanger. En conclusion, le phénotype adulte associé aux mutations de *CYP24A1* peut mimer une hyperparathyroïdie avec, cependant, des concentrations peu élevées de PTH, et un tableau d'adénome ou d'hyperplasie modérée des parathyroïdes. La chirurgie permet de diminuer la calciurie et d'améliorer la fonction rénale. Le cinacalcet est partiellement efficace sur l'hypercalcémie, dans la mesure où la PTH est inappropriée. Ce nouveau phénotype est une phénocopie d'hyperparathyroïdie, qui semble pouvoir se compliquer dans quelques cas d'authentique hyperparathyroïdie, bien qu'on ne puisse exclure une association accidentelle de ces 2 maladies.
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The only curative treatment of primary hyperparathyroidism is surgery. Nevertheless, hypercalcemia persists or reoccurs, in up to 20% of cases, leading to suspect multiglandular disease, ectopic location, parathyromatosis, parathyroid cancer, graft-dependent hypercalcemia and/or genetic causes. The *CYP24A1* gene belongs to the CYP450 group and encodes for 25-hydroxyvitamin D (25-OHD) 24-hydroxylase, a key enzyme of calcitriol (1-25-$(OH)_2$D) degradation. Reduced rates of inactivation of 1-25-$(OH)_2$D caused by a *CYP24A1* gene defect result in increased serum 1-25-$(OH)_2$D levels and intestinal absorption of calcium, causing downregulation of PTH secretion. The biological phenotype, characterized by hypercalcemia, hypercalciuria, depressed PTH, normal 25-OHD, increased 1-25-$(OH)_2$D, and decreased 24-25-$(OH)_2D_3$ levels [1–5], is characteristic of infantile hypercalcemia, which may also be related to *SLC34A1* gene mutations [6]. Late-onset forms include nephrolithiasis/-calcinosis, chronic renal insufficiency and hypertension [2–5,7–9], but the late outcome remains unclear.

Treatment includes hyperhydration and avoidance of sun and 25-OHD supplementation [10]. Steroids are ineffective [2,7]. Thiazides and sodium cellulose phosphate (a calcium intestinal absorption inhibitor) [5,7], pamidronate, and keto- or fluconazole (25-OHD-1-alpha-hydroxylase inhibitors) [2,5,7,11] can normalize calcium, though with possible liver toxicity and decreased adrenal function for ketoconazole. These treatments

http://dx.doi.org/10.1016/j.ando.2016.03.002
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Table 1
Pre- and post-surgery data of the 2 probands.

Patients	Normal range (units)	#1 (pre surgery 1)	#1 (6 weeks post surgery 1)	#2 (pre surgery)	#2 (4 years post surgery)
Gender	–	Female		Male	
Age at diagnosis	Years	39	40	51	54
BMI	18–25 kg/m^2	22	22	24	24
Phenotype					
Nephrolithiasis		+		+	
Nephrocalcinosis		+		+	
Hypertension		+		+	
Tendonitis		+		+	
T-score hip/spine		+1.20/+0.4	Not done	−1.7/−1.1	−1.9/−1.1
Biology					
Blood					
Calcium	2.15–2.55 mmol/L	3.47	3.27	2.62 to 2.87	2.42 to 2.90
Phosphate	0.81–1.45 mmol/L	0.90	0.70	0.80	0.80 to 0.90
Magnesium	0.75–1.2 mmol/L	0.78	0.69	–	0.82
Creatinine	53–106 μmol/L	105	114	124	123
FGR/MDRD	> 60 mL/mn/1.73 m^2	53	44	54	51
25-OHD	30–80 ng/mL	38	27	26	26
1-25-$(OH)_2D$	18–70 ng/mL	120	68	56	52
25-OHD_3/24-25-$(OH)_2D_3$	< 25 (LC–MS/MS)	–	> 100	–	168
PTH	15–65 pg/mL	92	62	9, 22 then 27	34
FGF 23	36–96 RU/mL	206	358	–	436
Bone alkaline phosphatasis	18–24 μg/L	10	8	–	8
Osteocalcin	8–39 ng/mL	36	26	–	29
Crosslaps	< 3250 pmol/L F < 8600 pmol/L M	1705	6646	–	3340
Urine calcium	< 7.5 mmol/24 h	10.8	7.3	10 to 18.7	11
Parathyroid investigation					
Cervical US		Hypoechoic 10 × 9 × 7 mm thyroid nodule		Right 12 mm parathyroid lesion	Right 13 × 8 mm hyperplasic parathyroid
Cervical CT scan (parathyroid hypertrophy)	mm	Left inferior 9 × 5 mm		Right 12 mm	Possibly left superior
MIBI scintigraphy (parathyroid uptake)		Negative		Right	Negative
Choline PET/CT scan; parathyroid uptake			Negative		Bilateral inferior
Genetic					
CYP24A1 gene			p.Cys380Arg/p.Leu409Ser		p.Leu335 Profs[a]11/p.Arg396Gln
CASR gene			No anomaly		p.Gln1011Glu
HRPT2 gene			c.1418-17C>G[b]		c.237+28 T>C[b]
MEN1 or CKD genes			No anomaly		No anomaly

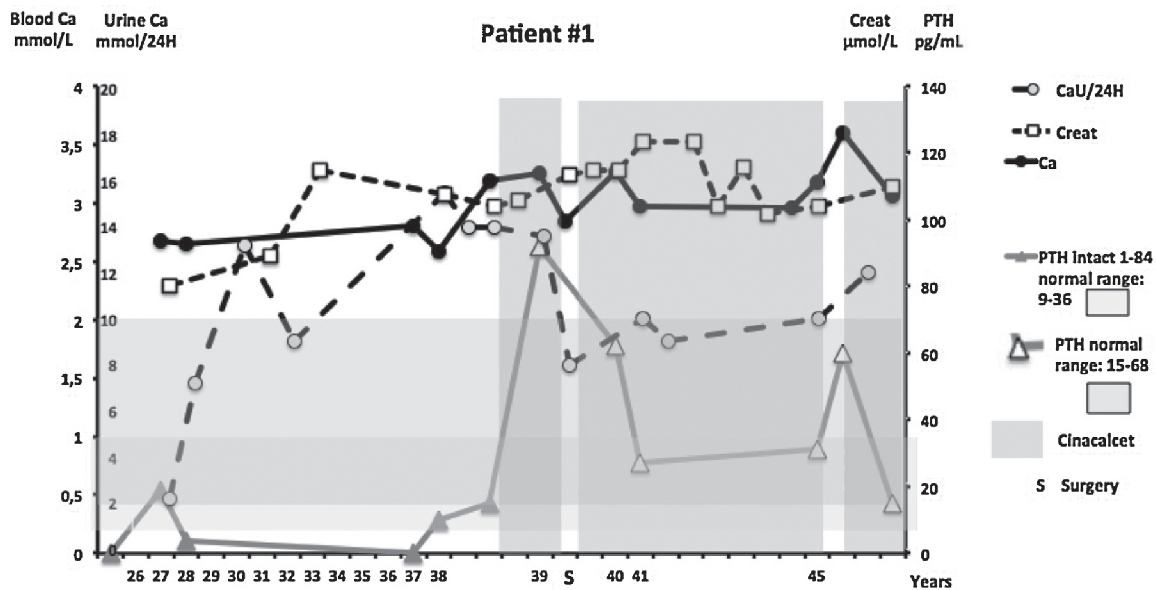
25-OHD: 25-hydroxyvitamin D; 1-25-$(OH)_2D_3$: calcitriol; 25-OHD_3/24-25-$(OH)_2D_3$: R ratio; 99mTc-methoxyisobutylisonitrile parathyroid scintigraphy: MIBI scintigraphy; BMI: body mass index; choline PET/CT scan: (^{18})F-methyl-choline positron emission tomography/computed tomography; CKD: cyclin dependent kinase; LC–MS/MS: liquid chromatography–tandem mass spectrometry; MEN1: menin; PTH: parathormone; US: ultrasound; T-score was measured with DEXA (dual-energy X-ray absorptiometry). When investigated, the 2 patients were treated with angiotensin receptor blockers, without any diuretics, and had normal conversion enzyme, PTH-related-peptide and CaSR antibody levels.
[a] Deleterious according to pathogenicity prediction programs (PolyPhen-2, Align-GVGD, MutationTaster, SIFT).
[b] Intronic polymorphism.

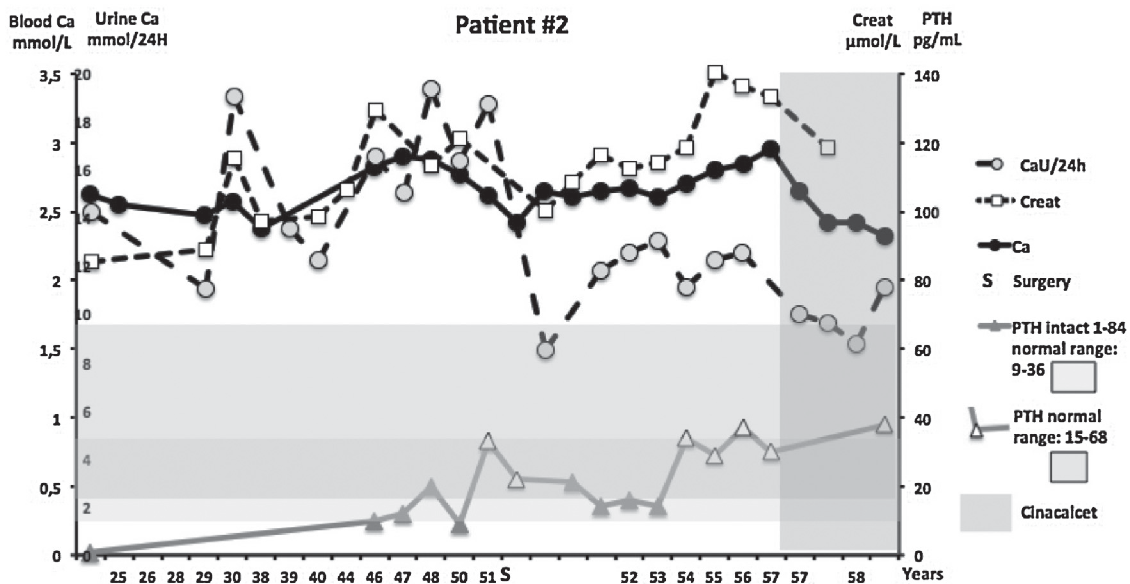
have focused on limiting the intestinal absorption of calcium since PTH level is depressed. The aim of this study was to report two adult patients with an unusual presentation mimicking hyperparathyroidism and their treatment with surgery and cinacalcet since PTH was increased.

Two unrelated adult patients were referred for nephrolithiasis since their twenties, tendonitis and hypertension, associated with blood calcium above 2.75 mmol/L, phosphate levels in the low normal range, hypercalciuria > 10 mmol/24 h despite slightly increased creatininemia, normal 25-OHD and inappropriate PTH (22 to 92 pg/mL; N: 15–68) levels, which had progressively increased with time (Table 1 and Fig. 1). Primary hyperparathyroidism was suspected.

Patient #1 had a family history of nephrolithiasis, breast cancer, and colonic polyps. Her cervical ultrasound and Tc-MIBI-scintigraphy showed a centimeter-sized intra-thyroid nodule, impossible to reach through fine needle aspiration, and a left MIBI-uptake concordant with left superior parathyroid hypertrophy on CT scan. Extensive surgical neck exploration was only able to locate 3 parathyroid glands, leading to the resection of 2 of them, associated to thymectomy and left thyroid lobo-isthmectomy because of the intra-thyroid nodule in case of an ectopic gland. Pathological examination showed hyperplasia of the 2 superior parathyroid glands and 3 papillary thyroid microcarcinomas (pT3mN0). Patient #2 had no special familial history. His cervical echography, Tc-MIBI-scintigraphy and tomodensitometry showed a 10 mm right parathyroid lesion corresponding to an adenoma after mini-surgery.

Level of 1-25-$(OH)_2D$ was normal or high. 25-OHD_3/24-25-$(OH)_2D_3$ ratio was overtly increased [12]. After written





informed consent, deleterious compound heterozygous mutations of the *CYP24A1* gene were identified (Table 1). In patient #1, a heterozygous intronic polymorphism of the *HRPT2* gene, without any mutation of the *CASR* gene, was otherwise identified; in patient #2, a well-known *CASR* gene variant and another intronic *HRPT2* gene polymorphism were found (Table 1). None of the patients showed *MEN1* or *CDK* genes mutation.

Hypercalciuria and kidney function improved post-surgery despite persistent hypercalcemia and inappropriate PTH levels, which led to the introduction of cinacalcet, enabling a decrease of creatininemia, calcemia, calciuria, and PTH levels (Table 1 and Fig. 1). Because of poor tolerance, patient #1, who had already had a poorly tolerated attempt of cinacalcet treatment before surgery, stopped the treatment during a month leading to a peak of calcemia (3.6 mmol/L), mirroring a transient increase of PTH level (Fig. 1). Parathyroid morphological re-investigation including cervical ultrasound, Tc-MIBI-scintigraphy and 18-fluoro-glucose-choline-PET/CT examination to discard a possible ectopic gland, remained negative. In patient #2, the cervical ultrasound showed a normal thyroid with possible right hyperplastic parathyroid gland, discordant with CT scan. MIBI-Tc-scintigraphy was normal but 18-fluoro-glucose-choline-PET/CT showed abnormal bilateral parathyroid uptake, arguing for eutopic at least hyperplastic and/or hyperfunctional parathyroid tissue.

The phenotypes of these 2 *CYP24A1*-mutated adult patients show that the biological profile might evolve over time from hypercalcemia with suppressed PTH levels towards a profile suggesting hyperparathyroidism with either a normal but inappropriate or a moderately increased PTH level. Therefore, recording the whole biological history of patients is mandatory. An early history of nephrolithiasis, tendonitis, initially suppressed PTH levels should prompt 25-OHD, 1-25-$(OH)_2D$ and 25-OHD_3/24-25-$(OH)_2D_3$ measurements, with *CYP24A1* gene investigation if this last ratio is increased above 25.

It is still unclear why these patients may develop true parathyroid hyperplasia and/or adenoma and whether surgery can provide improvement. In a closed loop, high 1-25-$(OH)_2D$ levels decrease both the secretory function and the volume of the parathyroid glands, inducing the expression of FGF23 in bone, which in turn induces the kidney expression of CYP24A1 to limit the 1-25-$(OH)_2D$ levels. In contrast, in chronic renal insufficiency, downregulation of the 25-OHD and calcium-sensing receptors in the parathyroid glands leads to hyperphosphatemia, hypocalcemia, and vitamin D deficiency, inducing parathyroid hyperplasia, and usually justifying subtotal parathyroidectomy. In our two patients, the blood phosphate levels, an independent factor of parathyroid hyperplasia, remained low despite the mild renal insufficiency, in relationship with a high FGF23 level. An incomplete surgery was suspected but morphological repeated investigations including ^{18}F-fluorocholine-PET/CT [13] did not provide further information in patient #1 who had had a subtotal parathyroidectomy, but showed two cervical foci, arguing for parathyroid hyperplasia in patient #2. Therefore, the adult phenotype of *CYP24A1* mutations could result from the balance between vitamin D excess related to the *CYP24A1* mutation which induces a reduction of parathyroid volume/secretion, while renal insufficiency related to hypercalciuria induces secondary hyperparathyroidism.

Another genetic cause could be suspected. Indeed, *cASR* gene mutations, is usually detected in patients with mild hypercalcemia, low urine calcium and normal to high PTH levels, and can be associated with parathyroid adenomas or hyperplasia in some cases. In this situation, adenoma resection usually induces a durable decrease in blood calcium, even if calcemia does not strictly return to the normal range; it was not the case here. In our two patients, neither *CASR* nor *MEN1, CDK*, *HRPT2* gene mutations were found, except for well-known sequence variants, which have never been involved in parathyroid adenoma or hyperplasia. A random association of *CYP24A1* mutation with hyperparathyroidism cannot be excluded. Therefore, *CYP24A1* mutations may not be added to the list of genetic forms of hyperparathyroidism related to loss of a tumor suppressor gene (*MEN1, CDC73/HRPT2*, *CDKN1B*), activation of *RET* proto-oncogen, and *CASR* gene mutation.

Interestingly, in patient #1, multiple microPTCs were identified. A relationship is suspected between these mutations and carcinogenesis [14,15].

Finally, besides lifestyle counseling and fluconazole, cinacalcet has been proposed as treatment for reducing high blood and urine calcium levels in symptomatic *CYP24A1*-mutated patients with normal or high PTH levels. In rare cases with evolution towards an inappropriate PTH level associated to positive morphological investigations, parathyroid surgery has been proposed mainly to decrease urine calcium level and stabilize or even improve kidney function, if medical treatment is not effective or not tolerated. If performed, surgery should include complete cervical exploration and subtotal parathyroidectomy, as for most genetic diseases.

To conclude, this adult phenotype of *CYP24A1* mutations is important to identify in order to adjust the treatment in atypical hyperparathyroidism with incipient nephropathy. This novel phenotype is a phenocopy of hyperparathyroidism, which might evolve in few cases towards hyperparathyroidism, despite the fact that random association of the 2 diseases cannot be excluded.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgement

The authors warmly thank G. Jones for having performed the R ratio.

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Joint Bone Spine 84 (2017) 349–351

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Case report

Calcium pyrophosphate deposition disease revealing a hypersensitivity to vitamin D

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ARTICLE INFO

Article history:
Accepted 8 November 2016
Available online 18 January 2017

Keywords:
Hypersensitivity to vitamin D
Calcium pyrophosphate deposition disease
Hypercalcemia
CYP24A1 mutation

ABSTRACT

Objective: Hypersensitivity to vitamin D (HVD) due to a loss of function mutation of the *CYP24A1* gene, which encodes vitamin D catabolizing enzyme was initially described as a cause of acute hypercalcemia in children and chronic renal diseases in adults.
Methods: We describe the first case of a patient presenting a calcium pyrophosphate deposition disease (CPDD) revealing a HVD.
Results: An abnormality of phospho-calcic metabolism was discovered during the course of an etiological workup for CPDD in a 52-year-old patient. Laboratory tests revealed a blood calcium level at the upper limit of normal range, a markedly low parathormone level, a 25-hydroxyvitamin D level within the upper level of normal, an elevated 1,25-dihydroxyvitamin D level and an elevated urine calcium level. *CYP24A1* gene sequencing analysis revealed two mutations in a heterozygous state. The study of the 25-hydroxyvitamin D_3: 24,25-dihydroxyvitamin D_3 ratio, two metabolites of vitamin D confirmed the enzyme deficiency in vivo. Our observation suggests that this disease could correspond to a rare cause of CPDD.
Conclusion: In cases of CPDD associated with calcium values within the upper limit of normal range (or hypercalcemia) with an abnormally low PTH, one could suggest searching for HVD.
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1. Introduction

Hypersensitivity to vitamin D (HVD), due to a deficit in vitamin D 24-hydroxylase (a catabolic enzyme of 1,25-dihydroxyvitamin D or calcitriol), is an autosomal recessive genetic disease linked to loss of function mutations of the *CYP24A1* gene. The disease is responsible for an accumulation of vitamin D with a dysregulation of intestinal calcium absorption and is characterized biologically by a tendency towards hypercalcemia with low PTH. Clinical manifestations of the disease in children are essentially those of acute hypercalcemia (dehydration, polyuria-polydipsia, nervousness, vomiting). In adults, it is characterized by complications caused by chronic renal hypercalciuria associated with nephrocalcinosis or renal lithiasis.

We describe the case of a patient presenting a calcium pyrophosphate deposition disease revealing a hypersensitivity to vitamin D.

2. Clinical presentation

A 52-year-old patient was hospitalized in the rheumatology department for investigation of an abnormal phospho-calcic metabolism discovered during the course of an etiological workup for calcium pyrophosphate deposition disease (CPDD), which had been progressing since the age of 39 years. He had a typical radiographic articular chondrocalcinosis, pubis radiograph showed calcium deposition in the pubic symphysis (Fig. 1), wrist radiograph showed calcium deposition in the triangular ligament of the right carpus (Fig. 2), knee radiograph showed bilateral calcium deposition in medial and lateral menisci (Fig. 3).

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http://dx.doi.org/10.1016/j.jbspin.2016.11.006
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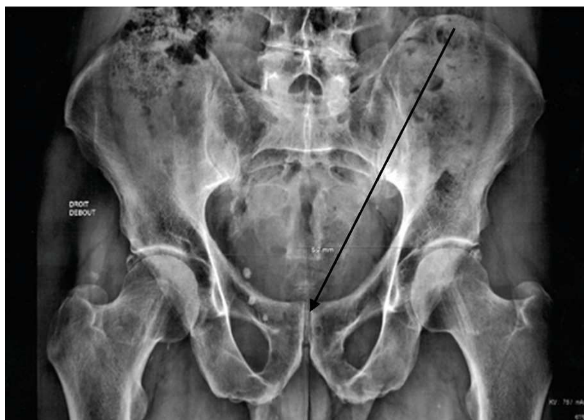

Fig. 1. Calcium deposition in the pubic symphysis.

The patient's past medical history included a monoclonal gammopathy of IgG lambda type and densitometric osteoporosis (T-score −2.91 SD at the lumbar spine, −1.68 SD at the femoral neck). Other medical history included a neurogenic thoracic outlet syndrome with previous surgical intervention and an obstructive sleep apnea syndrome. The patient had no past history of renal disease. The clinical exam was unremarkable, the general condition of the patient was good, and there was no axial or peripheral joint involvement. There was no tumoral syndrome at the clinical evaluation.

Laboratory tests revealed a blood calcium level at the upper limit of normal range (2.6 mmol/L), a markedly low parathormone (PTH) level (6.8 ng/mL), a 25-hydroxyvitamin D level within the upper level of normal although the patient had not received any vitamin D supplement (153.3 nmol/L), an elevated 1,25-dihydroxyvitamin D level (194 pmol/L; normal range, 48–110) as well as an elevated urine calcium level of 8.74 mmol/24 h.

Complementary exploration showed no evidence in favor of granulomatosis (angiotensin converting enzyme and biopsy of the accessory salivary glands were normal), malignant hemopathy (complete blood count normal, plasma protein electrophoresis showed a stable monoclonal peak, sternal bone marrow aspiration

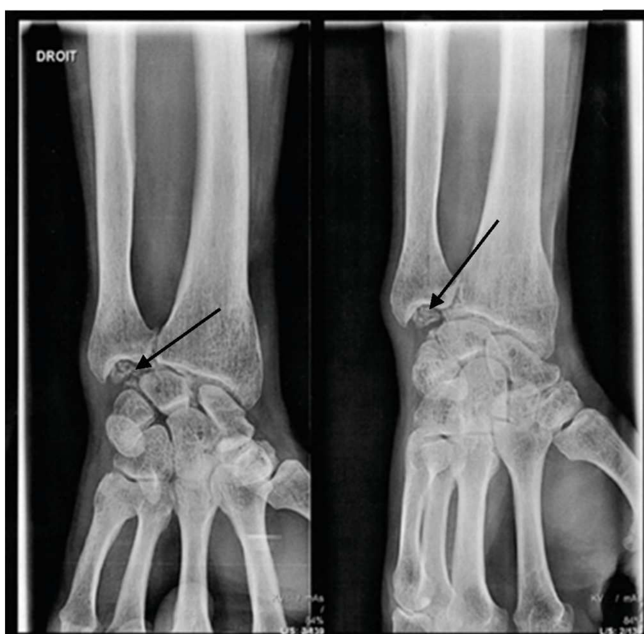

Fig. 2. Right wrist radiograph showing triangular cartilage calcification.

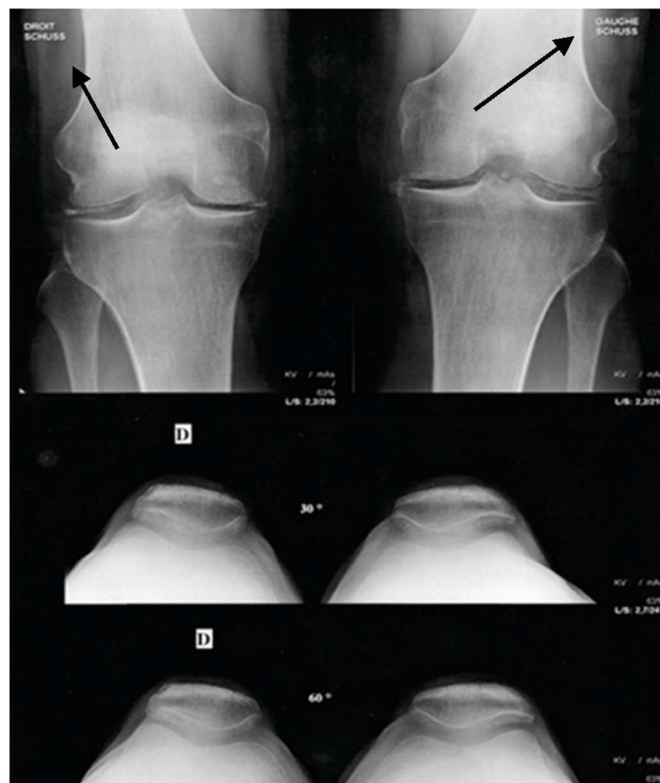

Fig. 3. Knee radiograph showing bilateral meniscal calcifications.

normal), or neoplasia (chest-abdomino-pelvic CT scan and PET-CT scan normal). HLA-B27 typing was positive. The presence of nephrocalcinosis or nephrolithiasis was excluded by the abdomino-pelvic CT scan.

HVD due to a vitamin D 24-hydroxylase deficiency was thus suspected and the patient was given a genetic consultation and a *CYP24A1* gene sequencing analysis. The result revealed the c.427_429del (p.Glu143del) recurrent deletion and a c.62delC (p.Pro21Argfs*8) frameshift mutation, both in a heterozygous state. The study of the 25-hydroxyvitamin D_3: 24,25-dihydroxyvitamin D_3 (25-OH-D_3/24,25-$(OH)_2$-D_3) ratio, two metabolites of vitamin D measured by tandem liquid chromatography coupled with mass spectrometry (LC-MS/MS), confirmed the enzyme deficiency in vivo (25-OH-D_3 = 110.8 nmol/L, 24,25-$(OH)_2$-D = 0.7 nmol/L, ratio = 158).

3. Discussion

To our knowledge, this is the first observation of CPDD associated with HVD due to a vitamin D 24-hydroxylase deficiency that has been documented clinically, radiologically, biologically, and genetically. We have found no publication reporting an association between these two abnormalities; the body of published works to date discusses metabolic and renal consequences of HVD found mostly in children.

Calcium pyrophosphate deposition disease is a crystal-related arthropathy and one of the most common inflammatory joint disease. It is characterized by the presence of calcium pyrophosphate dihydrate crystals within the cartilage of the joints and fibrocartilage [1,2]. The disease is frequent in the elderly, is most often idiopathic, and is not usually found in young persons in which it can occur as secondary to a metabolic disorder [3].

Hence, the most frequent causes of CPDD are primary hyperparathyroidism, haemochromatosis, and hypomagnesemia [4].

Rarely, genetic causes have been described based on observational studies of databases or clinical case reports. These include familial hypocalciuric hypercalcemia (mutation of the *CASR*) gene [5], familial hypomagnesemia with hypercalciuria and nephrocalcinosis (mutation of the *CLDN16* gene) [6], and familial calcium pyrophosphate deposition disease (mutation of the *ANKH* gene) [7].

Mutations of the *CYP24A1* gene causing hypersensitivity to vitamin D were not identified until recently in children presenting infantile idiopathic hypercalcemia [8]. Since then, there are several clinical cases in adults and a series of adults patients with essentially kidney complications [9–12].

Our observation suggests that this disease could correspond to a rare cause of CPDD. Similar to chronic hypercalcemia resulting from hyperparathyroidism, chronic hypercalcemia linked to HVD could inhibit the chondrocyte alkaline phosphatase and increase the extracellular inorganic pyrophosphate (ePPi) and the formation and deposition of calcium pyrophosphate dihydrate crystals and inflammatory joint disease. It is nevertheless not scientifically sound to state a causality between HVD and CPDD based on only one observation, and other works will be necessary to confirm or dispel this physiopathological hypothesis.

A parathormone level is part of an etiological workup routinely performed for CPDD to detect hyperparathyroidism. Conversely, in cases of association of calcium values within the upper limit of normal range (or hypercalcemia) with an abnormally low PTH, and normal or limit of normal values (or increased) of 25-OH-D_3 and 1,25-$(OH)_2$-D_3, and after exclusion granulomatosis and neoplasias, we would suggest to search for a *CYP24A1* mutation and perhaps to evaluate the 25-OH-D_3/24,25-$(OH)_2$-D_3 ratio [12]. If tests are found positive, the contra-indication of vitamin D supplementation will help to limit the risk of acute and chronic hypercalcemia. The exam is performed on a blood specimen taken after obtaining written informed consent from the patient for a genetic study.

Disclosure of interest

The authors declare that they have no competing interest.

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Improved Screening Test for Idiopathic Infantile Hypercalcemia Confirms Residual Levels of Serum 24,25-$(OH)_2D_3$ in Affected Patients

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ABSTRACT

CYP24A1 mutations are now accepted as a cause of idiopathic infantile hypercalcemia (IIH). A rapid liquid-chromatography tandem mass spectrometry (LC-MS/MS)-based blood test enabling measurement of the 25-OH-D_3:24,25-$(OH)_2D_3$ ratio (R) can identify IIH patients on the basis of reduced C24-hydroxylation of 25-OH-D_3 by CYP24A1 in vivo. Although values of this ratio are significantly elevated in IIH, somewhat surprisingly, serum 24,25-$(OH)_2D_3$ remains detectable. The current study explores possible explanations for this including: residual CYP24A1 enzyme activity in individuals with certain *CYP24A1* genotypes, expression of alternative C24-hydroxylases, and the possibility of isobaric contamination of the 24,25-$(OH)_2D_3$ peak on LC-MS/MS. We employed an extended 20-min run time on LC-MS/MS to study serum vitamin D metabolites in patients with IIH due to mutations of *CYP24A1* or *SLC34A1*; in unaffected heterozygotes and dialysis patients; in patients with vitamin D deficiency; as well as in normal subjects exhibiting a broad range of 25-OH-D levels. We identified 25,26-$(OH)_2D_3$ as a contaminant of the 24,25-$(OH)_2D_3$ peak. In normals, the concentration of 24,25-$(OH)_2D_3$ greatly exceeds 25,26-$(OH)_2D_3$; however, 25,26-$(OH)_2D_3$ becomes more significant in IIH with *CYP24A1* mutations and in dialysis patients, where 24,25-$(OH)_2D_3$ levels are low when CYP24A1 function is compromised. Mean R in 30 IIH-CYP24A1 patients was 700 (range, 166 to 2168; cutoff = 140) as compared with 31 in 163 controls. Furthermore, patients possessing *CYP24A1* L409S alleles exhibited higher 24,25-$(OH)_2D_3$ levels and lower R (mean R = 268; $n = 8$) than patients with other mutations. We conclude that a chromatographic approach which resolves 24,25-$(OH)_2D_3$ from 25,26-$(OH)_2D_3$ produces a more accurate R that can be used to differentiate pathological states where CYP24A1 activity is altered. The origin of the residual serum 24,25-$(OH)_2D_3$ in IIH patients appears to be multifactorial. © 2017 American Society for Bone and Mineral Research.

KEY WORDS: CYP24A1; HYPERCALCEMIA; 24,25-$(OH)_2D_3$; 25,26-$(OH)_2D_3$; LC-MS/MS

Introduction

The discovery of patients with idiopathic infantile hypercalcemia (IIH) due to genetic mutations of *CYP24A1* (25-OH-D_3-24-hydroxylase)[1] and *SLC34A1* (NaPi-IIa cotransporter)[2] represent events that have opened the door for defining the underlying molecular mechanism for the hypercalcemia in a significant percentage of the patients with IIH. In the case of mutations of *CYP24A1*, the hypercalcemia is due to the loss of the CYP24A1 enzyme responsible for the multistep catabolism of the side chain of the active form of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) and its precursor 25-hydroxyvitamin D_3 (25-OH-D_3),[3] which leads to elevations of these vitamin D metabolites in the blood.[1,4]

Though genotyping of these calcium and phosphate-related genes is critical for arriving at a correct diagnosis in IIH, the measurement of the main serum catabolite, 24,25-dihydroxy vitamin D_3 (24,25-$(OH)_2D_3$), can be helpful as a screening tool in rationalizing the deployment of these expensive genetic tests. We have devised a rapid liquid-chromatography tandem mass spectrometry (LC-MS/MS)-based assay that measures the concentration of several vitamin D metabolites simultaneously

Received in original form September 27, 2016; revised form February 22, 2017; accepted March 15, 2017. Accepted manuscript online March 17, 2017.
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Additional Supporting Information may be found in the online version of this article.

Journal of Bone and Mineral Research, Vol. 32, No. 7, July 2017, pp 1589–1596
DOI: 10.1002/jbmr.3135
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in a 100-μL serum sample and provides a 25-OH-D_3:24,25-$(OH)_2D_3$ ratio, which when elevated to values >80 (normal ratio, 5 to 25) allows for the identification of IIH due to a loss-of-function *CYP24A1* mutation.[5] Thus far, in our hands, this assay has been 100% successful in predicting the presence of loss-of-function *CYP24A1* mutations in individuals who have been subsequently shown by genotyping to have two affected *CYP24A1* alleles.[1,4] However, the assay still detects a small residual amount of 24,25-$(OH)_2D_3$ in most, if not all, patients tested, so that the 25-OH-D_3:24,25-$(OH)_2D_3$ ratio remains around 100 to 150. Similarly, others using either LC-MS/MS[6,7] or non-LC-MS/MS–based assays[8] have also detected a residue of 24,25-$(OH)_2D_3$ in IIH patients.

Consequently, we have asked the questions: Why do the levels of 24,25-$(OH)_2D_3$ not fall to undetectable values in IIH patients? and thus: Why does the 25-OH-D_3:24,25-$(OH)_2D_3$ ratio not become infinity in IIH patients where the CYP24A1 protein is devoid of enzyme activity? There are several possible answers, or combinations thereof, to this question, including the following: (1) another human CYP isoform exists that can synthesize small amounts of 24,25-$(OH)_2D_3$; (2) mutant CYP24A1 proteins retain some residual 24-hydroxylase enzyme activity; and (3) the putative 24,25-$(OH)_2D_3$ peak in LC-MS/MS contains small amounts of other vitamin D metabolites which remain unresolved under current LC-MS/MS conditions.

Although there is some evidence to support explanation #1, namely that the abundant and versatile liver CYP3A4 produces small amounts of 24,25-$(OH)_2D_3$ in experiments in vitro,[9] no data exits to support that it can synthesize this metabolite in vivo. Explanation #2 is feasible in the case of certain missense mutations of CYP24A1, but would probably not explain the data in patients with premature stop codons or frameshift mutations such as those found by Schlingmann and colleagues.[1] Furthermore, the *Cyp24a1*-null mouse[10] with a complete absence of the critical heme-binding domain also shows a small residual "putative 24,25-$(OH)_2D_3$ peak."[11] Thus, on the face of it, explanation #3 appears the most logical one, especially when one considers that the *Cyp24a1*-null mouse has a complete absence of another rodent-specific metabolic product of CYP24A1, namely 25-hydroxyvitamin D_3-26,23-lactone (25-OH-D_3-26,23-lactone).[11]

Moreover, we reasoned that we could test the identity of the "putative 24,25-$(OH)_2D_3$ peak" in IIH patients by use of more rigorous chromatographic conditions during LC-MS/MS. We report here our use of extended chromatography to better resolve the 24,25-$(OH)_2D_3$ peak from potential interferences, not only in IIH patients with confirmed *CYP24A1* biallelic mutations,[1,4] but also in their heterozygotic siblings/relatives[4]; in a normal population with a range of 25-OH-D_3 values[12–14]; and in patients with chronic kidney disease (CKD) who are on dialysis, because these individuals show higher 25-OH-D_3:24,25-$(OH)_2D_3$ ratios than normal using the existing published method.[15,16]

Materials and Methods

Clinical samples

Clinical samples were collected as part of several different ethically-approved investigations of patients with idiopathic infantile hypercalcemia reported previously by Schlingmann and colleagues[1,2] and Molin and colleagues[4] (mutations listed in Supplemental Material); dialysis patients with CKD stage 5 (glomerular filtration rate [GFR] <15) in studies reported by Armas and colleagues[15,16]; and normal premenopausal and postmenopausal women coordinated and reported by Kaufmann and colleagues[5] and Gallagher and colleagues.[12–14] *Cyp24a1*- null and *vdr*-null mouse serum samples[10] were provided by Drs. J Wesley Pike (University of Wisconsin–Madison, Madison, WI, USA) and Rene St-Arnaud (McGill University and Shriner's Hospital, Montreal, QC, Canada).

LC-MS/MS of vitamin D metabolites

Vitamin D metabolites [25-OH-D_3, 25-OH-D_2, 3-epi-25-OH-D_3, and 24,25-$(OH)_2D_3$] were measured simultaneously using 100 μL serum with a novel, very sensitive LC-MS/MS–based method involving derivatization with DMEQ-TAD (Key Synthesis, Oaks PA, USA); also known as 4-(2-(6,7-Dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl)-1,2,4-triazoline-3,5-dione).[5] Briefly, this method involved the addition of commercially-available, deuterated internal standards, including d_6-24,25-$(OH)_2D_3$, for each metabolite quantified; zinc sulfate protein precipitation; and a liquid-liquid extraction using hexane-methyl tertiary butyl ether. LC-MS/MS was performed on triplicate injections of 10-μL aliquots of each serum extract (each representing 16-μL equivalents of serum) using an Acquity ultra performance liquid chromatography (UPLC) connected in-line with a Xevo TQ-S mass spectrometer in electrospray positive mode (Waters Corp., Milford, MA, USA). Chromatography was achieved using a ethylene bridged hybrid [BEH]-Phenyl UPLC column (1.7 μm, 2.1 × 50 mm) (Waters Corp.) and a 5-min methanol/water–based gradient system in order to separate the DMEQ-TAD derivatives of the various vitamin D metabolites. DMEQ-TAD forms two adduct peaks from each vitamin D metabolite in the serum sample and the two adducts are usually resolved on LC-MS/MS. Using this gradient system the two DMEQ-TAD peaks of 24,25-$(OH)_2D_3$ elute at 1.6 and 2.3 min, with the latter peak being utilized for quantification.

Extended chromatography LC-MS/MS

Serum samples (50 μL) were extracted and derivatized as described,[5] dried extracts reconstituted in a volume of 50 μL mobile phase and 35 μL of extract (equivalent to 35 μL serum) was injected onto the LC column. LC-MS/MS analysis was performed as before using an Acquity UPLC connected in-line with a Xevo TQ-S mass spectrometer in electrospray positive mode (Waters Corp.). The LC method employed a methanol/water–based gradient system, where mobile phase B comprised methanol supplemented with 0.1% formic acid and 2 mM ammonium acetate, and mobile phase A comprised of water also supplemented with 0.1% formic acid and 2 mM ammonium acetate. Separations were achieved using a Cortecs C18+ column (2.1 × 100 mm; 1.6 μm) at a flow rate of 0.350 mL/min. An initial composition of 50% mobile phase B was brought to 77% over an 18-min linear gradient. The shallower gradient system results in elution of DMEQ-TAD adducts of 24,25-$(OH)_2D_3$ at 9.2 and 11.2 minutes and resolved from other dihydroxyvitamin D_3 metabolites (Supplemental Material, Supplemental Fig. 1). DMEQ-TAD derivatives of synthetic 25S,26-$(OH)_2D_3$ (a generous gift of Dr. Milan Uskokovic, Hoffman La Roche, Nutley, NJ, USA)[17] eluted at 9.90 and 11.39 min; and DMEQ-TAD derivatives of synthetic 1,25-$(OH)_2D_3$ eluted at 12.15 and 13.20 min. In the case of extended chromatography, quantification of 24,25-$(OH)_2D_3$ was based upon either of its DMEQ-TAD adducts, in comparison to their respective d_6-24,25-$(OH)_2D_3$ internal standard peaks. Concentration of the metabolite

$25,26\text{-}(OH)_2D_3$ was estimated based on the calibration line for $24,25\text{-}(OH)_2D_3$ and recovery of $d_6\text{-}24,25\text{-}(OH)_2D_3$, because there was no $d_6\text{-}25,26\text{-}(OH)_2D_3$ internal standard available for our study. Assay performance characteristics, $24,25\text{-}(OH)_2D_3$ calibration line, and relative retention times of vitamin D standards are shown in the Supplemental Material.

Statistical analyses

Statistics were generated using Prism (GraphPad Software, Inc., La Jolla, CA, USA). Results are displayed as the mean ± SD and significant differences were determined using the Student's *t* test.

Results

The extended chromatography approach resolved the major adduct peaks of $24,25\text{-}(OH)_2D_3$, $25,26\text{-}(OH)_2D_3$, and $1,25\text{-}(OH)_2D_3$, thereby enabling us to determine the composition of the "putative $24,25\text{-}(OH)_2D_3$ peak" in a range of clinical and *Cyp24A1*-null mouse serum samples. When we subjected serum extracts from IIH patients or *Cyp24a1*-null mice to sodium m-periodate treatment[18] prior to DMEQ-TAD derivatization and LC-MS/MS, the dihydroxyvitamin D_3 traces were rendered devoid of the periodate-sensitive, vicinal dihydroxyvitamin D metabolites, $24,25\text{-}(OH)_2D_3$ and $25,26\text{-}(OH)_2D_3$ peaks, and only peaks corresponding to $1,25\text{-}(OH)_2D_3$ remained (data not shown).

In all IIH patients tested, the "putative $24,25\text{-}(OH)_2D_3$ peak" from the published method was resolved into one of the two adduct peaks for $25,26\text{-}(OH)_2D_3$ (at 11.40 min) (co-migrating with authentic $25S,26\text{-}(OH)_2D_3$) and one of the adduct peaks from $24,25\text{-}(OH)_2D_3$ (at 11.2 min) (Fig. 1*A*, Supplemental Material). In all IIH patients the residual $24,25\text{-}(OH)_2D_3$ was greater than the LOQ and limit of quantitation (LOQ) for the method and thus could not be considered an artifact (see Supplemental Material). However, in almost all 30 IIH patients, the size of the $25,26\text{-}(OH)_2D_3$ peak exceeded that of $24,25\text{-}(OH)_2D_3$. We observed that the relative sizes of the $24,25\text{-}(OH)_2D_3$ and $25,26\text{-}(OH)_2D_3$ peaks varied depending on the nature of the *CYP24A1* mutation. Patients with at least one L409S *CYP24A1* allele exhibited a higher proportion of $24,25\text{-}(OH)_2D_3$ than other *CYP24A1* mutations (example, Fig. 1*A*). This is depicted quantitatively by plotting the $25,26\text{-}(OH)_2D_3$:$24,25\text{-}(OH)_2D_3$ ratio where eight patients with L409S average 2.00 ± 0.81 (mean ± SD; range, 1.1 to 3.7) while all 22 patients with other CYP24A1 genotypes average 6.32 ± 3.23 (range, 0.61 to 18.73; $p = 0.001$) (Fig. 2*A*; Table 1). In a similar fashion, the $25\text{-}OH\text{-}D_3$:$24,25\text{-}(OH)_2D_3$ ratio in patients with L409S average 268 ± 70 (range, 165 to 403) while the remaining 22 IIH patients average 858 ± 436 (range, 322 to 2167; $p = 0.001$) (Fig. 2*B*; Table 1); whereas ratios determined for patients with L409S (105.51 ± 14.05) could not be differentiated from other genotypes (129.33 ± 43.50) using the published method ($p = 0.154$). In contrast, serum samples with a full range of 25-OH-D concentrations from normal premenopausal and postmenopausal women before and after treatment with various doses of vitamin D_3 (0 to 4800 IU/day for 1 year)[12–14] show a much lower $25,26\text{-}(OH)_2D_3$:$24,25\text{-}(OH)_2D_3$ ratio of 0.30 ± 0.22, where the most abundant component was $24,25\text{-}(OH)_2D_3$ along with only a small peak of $25,26\text{-}(OH)_2D_3$ ($p < 0.001$ as compared with IIH CYP24A1) (Fig. 1*B*). *CYP24A1* heterozygotes with one affected allele, as well as IIH patients with mutations of *SLC34A1*[2] exhibited a metabolite pattern and $25,26\text{-}(OH)_2D_3/24,25\text{-}(OH)_2D_3$ ratios of 0.23 ± 0.05 and 0.34 ± 0.14, respectively, that did not differ from all normals ($p = 0.124$ and $p = 0.649$, respectively) (Fig. 1*B*). On the other hand, serum samples from CKD stage 5 dialysis patients[15,16] show an abnormal $24,25\text{-}(OH)_2D_3/25,26\text{-}(OH)_2D_3$ pattern more comparable to IIH patients (Fig. 1*C*) and a $25,26\text{-}(OH)_2D_3$:$24,25\text{-}(OH)_2D_3$ ratio of 1.66 ± 1.07 (Table 1), which did not differ from the subset of IIH CYP24A1 patients with L409S ($p = 0.436$). A list of the genotypes of IIH-CYP24A1 patients presented in this study is provided in the Supplemental Material.

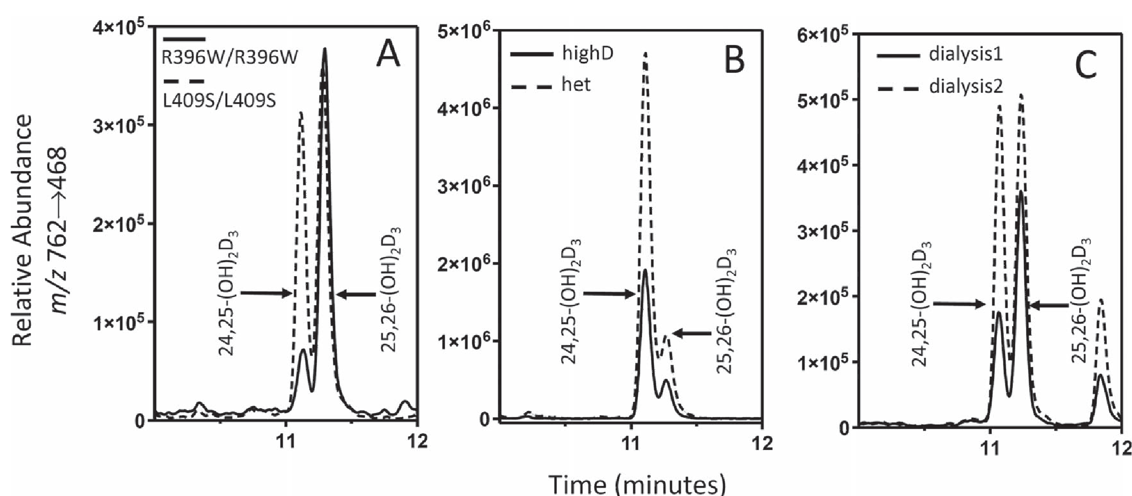

Fig. 1. LC-MS/MS of DMEQ-TAD derivatives of $24,25\text{-}(OH)_2D_3$ and $25,26\text{-}(OH)_2D_3$ using extended chromatography in different patient groups. Using gradient chromatographic conditions, described in the text, the derivatives of $24,25\text{-}(OH)_2D_3$ and $25,26\text{-}(OH)_2D_3$ emerge at 11.2 and 11.4 min, respectively. (*A*) Typical pattern observed in IIH patients with biallelic L409S mutations (dashed line) and biallelic R396W mutations (unbroken line); (*B*) typical pattern observed in CYP24A1 heterozygotic relatives of IIH patients (unbroken line); and in normal postmenopausal females with high 25-OH-D levels (>20 ng/mL)(dashed line) or low 25-OH-D levels (<20 ng/mL) (half-tone); (*C*) two examples of the patterns observed in dialysis patients.

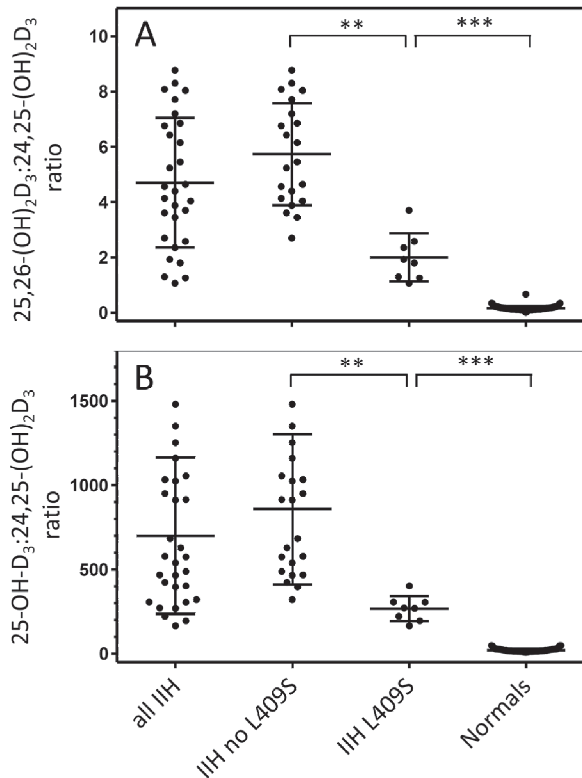

Fig. 2. (*A*) Ratio of 25,26-$(OH)_2D_3$:24,25-$(OH)_2D_3$; and (*B*) ratio of 25-OH-D_3:24,25-$(OH)_2D_3$ observed in different groups of IIH patients and normals. The ranges of 25,26-$(OH)_2D_3$:24,25-$(OH)_2D_3$ ratio and of 25-OH-D_3:24,25-$(OH)_2D_3$ are depicted for all 30 IIH patients studied (left); in the 8 IIH patients with one or more copy of the L409S mutation (middle left); in 22 IIH patients with other CYP24A1 mutations (middle right); or normals (right). Statistical significance between selected groups is denoted by $p = 0.001$ (**) and $p < 0.001$ (***).

The existence of two adduct peaks from 24,25-$(OH)_2D_3$ with DMEQ-TAD allowed for alternative methods for quantifying the components and, importantly, the 25-OH-D_3:24,25-$(OH)_2D_3$ ratio for IIH patients and their relatives. However, unlike other analysts[7] who had a similar problem separating 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) adducts of 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$, we chose the larger of the two adduct peaks (11.20 min) to quantitate 24,25-$(OH)_2D_3$ and determine the 25-OH-D_3:24,25-$(OH)_2D_3$ ratios used in this study (Table 1). Whereas the rapid chromatographic approach fails to resolve 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ and therefore overestimates 24,25-$(OH)_2D_3$ values and falsely lowers the 25-OH-D_3:24,25-$(OH)_2D_3$ ratio, the extended chromatography provides a fully resolved 24,25-$(OH)_2D_3$ peak and a more accurate 25-OH-D_3:24,25-$(OH)_2D_3$ ratio than with the published method (Fig. 2*B*; Fig. 3*A*, *B*). Using extended chromatography we have determined that an average of 80% of the dihydroxyvitamin D_3 component is 25,26-$(OH)_2D_3$ in IIH patients, as compared with 30% or 14% in normal subjects with 25-OH-D_3 <20 ng/mL or >20 ng/mL ($p < 0.001$), respectively, with the remainder comprising 24,25-$(OH)_2D_3$. As Table 1 and Fig. 3 show, the mean 25-OH-D_3:24,25-$(OH)_2D_3$ ratio in IIH patients increases significantly

Table 1. Mean Concentration and Ratios of Serum Vitamin D Metabolites in Various Patient Groups Determined Using Extended Chromatography LC-MS/MS

Patient group	n	25-OH-D_3 (ng/mL)	24,25-$(OH)_2D_3$ (ng/mL)	24,25-$(OH)_2D_3$ to 25-OH-D_3 ratio (1×10^{-2})	25-OH-D_3 to 24,25-$(OH)_2D_3$ ratio	p^a	25,26-$(OH)_2D_3$ (ng/mL)	25-OH-D_3 to 25,26-$(OH)_2D_3$ ratio	25,26-$(OH)_2D_3$ to 24,25-$(OH)_2D_3$ ratio	p^b
IIH CYP24A1 (all)	30	47.9 ± 18.0	0.09 ± 0.06	0.215 ± 0.138	700 ± 457	<0.001	0.38 ± 0.24	139 ± 37	5.17 ± 3.39	<0.001
IIH CYP24A1-L409S[c]	8	44.8 ± 14.0	0.16 ± 0.05	0.401 ± 0.107	268 ± 70	<0.001	0.32 ± 0.08	135 ± 26	2.00 ± 0.81	<0.001
IIH CYP24A1-no L409S	22	49.0 ± 19.1	0.07 ± 0.05	0.147 ± 0.069	858 ± 436	<0.001	0.40 ± 0.28	140 ± 40	6.32 ± 3.23	<0.001
IIH SLC34A1	6	34.9 ± 12.6	1.48 ± 1.24	3.660 ± 1.950	36 ± 19	0.513	0.38 ± 0.20	102 ± 20	0.34 ± 0.14	0.649
CYP24A1 (+/–)[d]	25	33.0 ± 13.8	1.57 ± 0.69	4.860 ± 1.590	24 ± 13	0.071	0.34 ± 0.11	106 ± 55	0.23 ± 0.05	0.126
Normals (all)	163	24.7 ± 18.9	1.30 ± 1.40	4.310 ± 2.250	31 ± 19	–	0.22 ± 0.19	116 ± 44	0.30 ± 0.22	–
Normals 25-D_3 >20 ng/mL	84	35.4 ± 19.4	2.07 ± 1.47	5.550 ± 2.110	21 ± 9	–	0.31 ± 0.22	132 ± 46	0.16 ± 0.08	–
Normals 25-D_3 <20 ng/mL	79	11.7 ± 5.2	0.35 ± 0.35	2.800 ± 1.290	43 ± 20	–	0.13 ± 0.05	97 ± 33	0.47 ± 0.22	–
Dialysis	24	25.7 ± 13.8	0.19 ± 0.29	0.730 ± 0.325	174 ± 14	<0.001	0.24 ± 0.15	108 ± 21	1.66 ± 1.07	<0.001

Values are mean ± SD.
[a]Value of p for t test based on comparison of 25-OH-D_3:24,25-$(OH)_2D_3$ ratios to all normal subjects.
[b]Value of p for t test based on comparison of 25,26-$(OH)_2D_3$:24,25-$(OH)_2D_3$ ratios to normal subjects.
[c]Subset of IIH CYP24A1 patients with at least one L409S allele.
[d]Heterozygous family members.

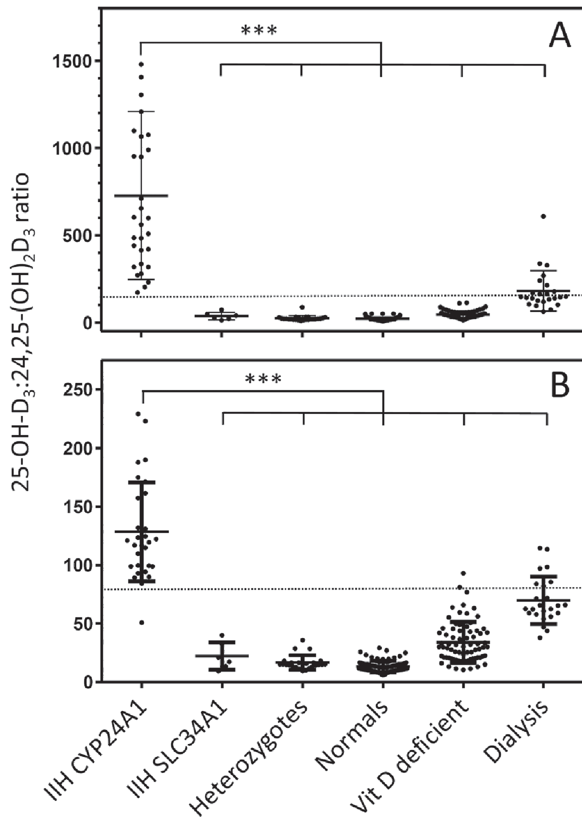

Fig. 3. Ratio of 25-OH-D_3:24,25-$(OH)_2D_3$ for several patient groups calculated based upon vitamin D metabolite data acquired using the two different LC-MS/MS methods. (*A*) The new extended chromatography method; (*B*) the published[(5)] rapid chromatography method. Note the increase in the values as a result of excluding 25,26-$(OH)_2D_3$ from the calculations in *A*. All patient samples were analyzed using both methods in the same laboratory. Proposed ratio cutoffs indicating the presence of IIH are shown as a dotted line, based on the extended (*A*: >140) or rapid (*B*: >80) chromatography approaches. Difference in ratios between IIH-CYP24Al patients and other clinical cohorts were statistically significant in all cases where $p < 0.001$ (***).

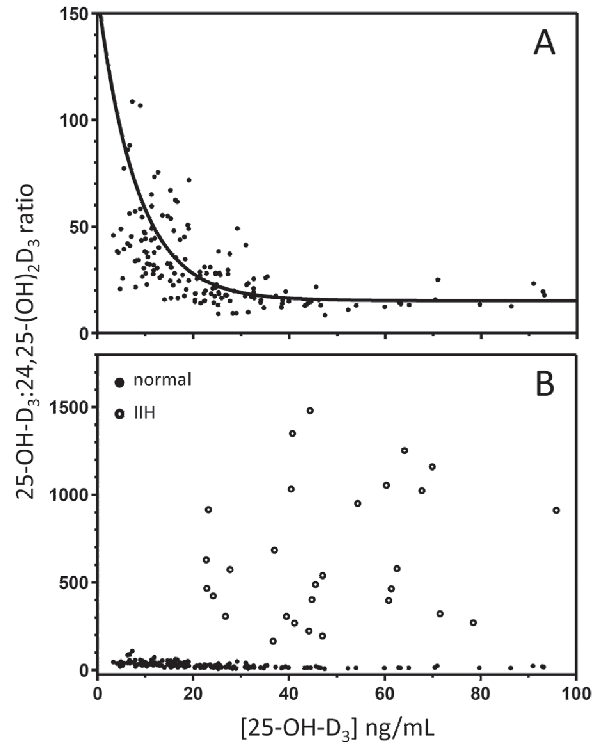

Fig. 4. Relationships between the 25-OH-D_3:24,25-$(OH)_2D_3$ ratio and 25-OH-D_3 over the full normal range of 25-OH-D_3 in a cohort of 156 normal premenopausal and postmenopausal women receiving graded doses of vitamin D_3 for 1 year. (*A*) Normal individuals (●) acquired using the new extended chromatography method; (*B*) the same normal individuals (●) plotted together with 30 IIH-CYP24A1 patients (○) on an expanded y-scale and showing the lack of overlap between the two groups.

from around 121 ± 39 using the published method to around 700 ± 457 (range, 166 to 2167; $p < 0.001$), whereas the ratio rises only marginally from 16 ± 6 to 24 ± 13 ($p = 0.010$) in CYP24A1 heterozygotes, where the most abundant dihydroxyvitamin D_3 component (81%) remains 24,25-$(OH)_2D_3$. Accordingly, we propose a 25-OH-D_3:24,25-$(OH)_2D_3$ ratio cutoff of 140 to indicate IIH under our current extended chromatography conditions.

As with the IIH heterozygotes, normal individuals given graded doses of 25-OH-D_3 also showed little change in the their 25-OH-D_3:24,25-$(OH)_2D_3$ ratios when analyzed using the extended chromatography methodology (Fig. 3*A*, *B*). Table 1 shows the range of 25-OH-D_3, 24,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$, and 25-OH-D_3:24,25-$(OH)_2D_3$ ratio values observed in these individuals. Though the mean 25-OH-D_3:24,25-$(OH)_2D_3$ ratios calculated using the new extended chromatography method increase from 21 ± 15 to 31 ± 19 ($p < 0.001$) in these normal individuals, they do not approach the very high values seen in IIH patients. Although, as seen before,[(5)] 25-OH-D_3:24,25-$(OH)_2D_3$ ratios rise dramatically as individuals become more vitamin D–deficient (Fig. 4*A*), none of these ratios ever overlap with the ratios observed in IIH patients (Fig. 4*B*). In contrast, patients on dialysis exhibit 25-OH-D_3:24,25-$(OH)_2D_3$ ratio values, which increase significantly from a mean of 67 ± 19 using the published method to 174 ± 14 using the new method ($p < 0.001$), presumably reflecting the decreased production of 24,25-$(OH)_2D_3$ when kidney function fails, rather than a response to the very high FGF23 values observed in dialysis patients (Table 1). Receiver operator characteristic (ROC) curves (Supplemental Fig. 2) indicate the improved ability of ratios determined using extended chromatography LC-MS/MS to differentiate IIH-CYP24A1 patients from vitamin D–deficient subjects, as well as IIH patients possessing at least one L409S allele from those with other genotypes. Although the mean 25-OH-D_3:24,25-$(OH)_2D_3$ ratios from the dialysis and IIH CYP24A1 L409S group differ significantly ($p = 0.035$), the overlap in ratios using either the rapid or extended chromatography approach suggest that our method will not be able to distinguish between IIH and dialysis patients in all cases. According to our results, likelihood analysis revealed that IIH-CYP24A1 patients were 34× more likely to possess a 25-OH-D_3:24,25-$(OH)_2D_3$ ratio >80 versus unaffected patients using the rapid chromatography approach, or 21× when using the extended chromatography approach where a 25-OH-D_3:24,25-$(OH)_2D_3$ ratio >140 was used as a cutoff. Although most of the overlap with ratios associated with IIH occurred in

a subset of dialysis patients, we emphasize that dialysis patients are likely to be identified primarily based on the distinct clinical course of chronic renal failure, rather than testing for vitamin D metabolites.

In normal individuals, serum levels of the metabolite 25,26-$(OH)_2D_3$ correlate with 25-OH-D_3 in the same way observed for 24,25-$(OH)_2D_3$ and 25-OH-D_3, as reported.[5,17] Although the plot of 24,25-$(OH)_2D_3$ versus 25-OH-D_3 shows an x axis intercept that suggests a downregulation of synthesis of 24,25-$(OH)_2D_3$ at low 25-OH-D_3 concentrations, the plot of 25,26-$(OH)_2D_3$ versus 25-OH-D_3 shows no intercept (Fig. 5*A*, *B*). Table 1 presents evidence that serum levels of 25,26-$(OH)_2D_3$ are not affected by IIH or by renal disease, suggesting that this metabolite is neither the product of CYP24A1 nor is it synthesized in the kidney. However, as expected, the data do show that levels of 25,26-$(OH)_2D_3$ decline in normal individuals at low 25-OH-D_3 values. Accordingly, vitamin D–deficient patients (with 25-OH-D_3 values $<$20 ng/mL) show a slight rise in the mean 25,26-$(OH)_2D_3$:24,25-$(OH)_2D_3$ ratio of 0.47 ± 0.22, but still show significantly lower values than those seen in IIH patients or in dialysis patients where there is a defect in the production of 24,25-$(OH)_2D_3$ (Table 1). Such data argues for a parallel decline in the synthesis of 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ in vitamin D–deficient patients due to a lack of the substrate, 25-OH-D_3.

Discussion

Use of extended chromatography that resolves 24,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ allows us further insights into the dysregulation of vitamin D metabolism in IIH patients. The putative "24,25-$(OH)_2D_3$ peak" previously found using a published rapid LC-MS/MS system[5] further resolves into two components: 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ on extended chromatography, revealing that IIH patients retain a small but measurable residue of 24,25-$(OH)_2D_3$. This finding is reinforced by the presence of two characteristic adduct peaks with DMEQ-TAD for both 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ in all IIH patients. Thus, the working hypothesis: that IIH patients have a residual "24,25-$(OH)_2D_3$ peak" because it is contaminated with another dihydroxyvitamin D_3 metabolite, is correct, but is only part of the full story. In comparison, serum from *Cyp24a1*-null mice,[10] when run on extended chromatography, also showed two adduct peaks for a minor 24,25-$(OH)_2D_3$ component and two adduct peaks for a major 25,26-$(OH)_2D_3$ component (data not shown).

The fact that there is a small amount of authentic 24,25-$(OH)_2D_3$ in all IIH patients with different mutations suggests either residual CYP24A1 enzyme activity in some mutants; or the existence of another 24-hydroxylase in addition to CYP24A1, a theory proposed and speculated on by others.[8] The fact that there is a cluster of IIH patients predominantly expressing one L409S *CYP24A1* allele with slightly higher 24,25-$(OH)_2D_3$ levels than the rest of the IIH patients; slightly lower, but abnormal, 25-OH-D_3:24,25-$(OH)_2D_3$ ratios; and with 25,26-$(OH)_2D_3$:24,25-$(OH)_2D_3$ ratios approaching unity, all point to the fact that *CYP24A1* with L409S retains some enzyme activity. Moreover, we concluded in an earlier publication[1] that the L409S mutant retains some residual activity when tested using an in vitro assay. Although it might be speculated that these IIH L409S patients have slightly less severe disease symptoms: nephrolithiasis and nephrocalcinosis, currently we find no major differences in the presentation of these patients.

Although some CYP24A1 mutant proteins may retain enzyme activity, this is almost certainly not true for all. In these situations, we favor that another 24-hydroxylase is operating and although experiments have suggested that other CYPs, eg, CYP3A4, can 24-hydroxylate vitamin D compounds in vitro,[9] until our study there was no evidence that this can occur in vivo. Furthermore, from the levels of 24,25-$(OH)_2D_3$ found in IIH patients, it appears that this unknown CYP is not very efficient and cannot offset the loss of CYP24A1 enzyme activity in the clearance of 25-OH-D_3 and 1,25-$(OH)_2D_3$, as judged by the resulting appearance of hypercalcemia. Presumably the CYP24A1-null mouse also expresses an alternative 24-hydroxylase to account for the residual 24,25-$(OH)_2D_3$ observed in the blood of this animal model too.

One consequence of the resolution of 24,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$, and a more accurate measure of 24,25-$(OH)_2D_3$ is a refined ratio of the metabolites, 25-OH-D_3 and 24,25-$(OH)_2D_3$. The data presented in Table 1 displays the ratio of these two metabolites both in the 24,25-$(OH)_2D_3$:25-OH-D_3 and 25-OH-D_3:24,25-$(OH)_2D_3$ formats. We prefer the latter format because it provides a convenient integer and we note that though there are small increases in this 25-OH-D_3:24,25-$(OH)_2D_3$ ratio for the other groups: vitamin D–deficient and dialysis patients, the more accurate 24,25-$(OH)_2D_3$ values obtained with the new longer method provide a better discrimination between IIH patients and these other vulnerable groups (Figs. 3 and 4), in particular those with vitamin D deficiency. Currently, the rapid LC-MS/MS method still provides a wide difference in 25-OH-D_3:24,25-$(OH)_2D_3$ ratios between IIH patients ($>$80) and normal individuals (5 to 25),[1,2,4] because 24,25-$(OH)_2D_3$ concentrations greatly exceed those of 25,26-$(OH)_2D_3$ in normal individuals and the latter metabolite is a minor component of the "putative 24,25-$(OH)_2D_3$ peak" in rapid analysis. This is supported by the relatively normal amounts of 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ determined here in *CYP24A1* heterozygotes, IIH patients with *SLC34A1* mutations and normal postmenopausal women observed in our studies. But we have shown that more accurate values for the 25-OH-D_3:24,25-$(OH)_2D_3$ ratio are attainable using the extended chromatography described herein, but with the price of a corresponding fourfold increase in analysis time. Nevertheless, we think that a 25-OH-D_3:24,25-$(OH)_2D_3$ ratio from this extended chromatography LC-MS/MS is a better screening tool for IIH patients and justifies the extra analysis time. Our work highlights the need to exercise caution when comparing 25-OH-D_3:24,25-$(OH)_2D_3$ ratios with cutoffs established by different laboratories and/or methods using a limited number of patients, because 25-OH-D_3:24,25-$(OH)_2D_3$ ratios vary significantly in IIH depending on whether 25,26-$(OH)_2D_3$ is separated from the 24,25-$(OH)_2D_3$ fraction, as well as according to CYP24A1 genotype. Two recently published reports on a limited number of IIH patients[6,7] paid careful attention to resolution of 25,26-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$, resulting in elevated 25-OH-D_3:24,25-$(OH)_2D_3$ ratios. Our study characterizes a 25-OH-D_3:24,25-$(OH)_2D_3$ ratio cutoff of 140 based on 30 IIH patients, the largest number of patients studied in a single laboratory that includes eight patients possessing the L409S genotype which exhibit lower 25-OH-D_3:24,25-$(OH)_2D_3$ ratios than other genotypes due to the presence of detectable yet still pathogenic levels of CYP24A1 enzyme activity associated with this mutation.

Despite 25,26-$(OH)_2D_3$ being one of the first dihydroxyvitamin D metabolites to be identified and studied in the late 1960s, its origins and role remain elusive. Although there have been a variety of different approaches (competitive protein-binding

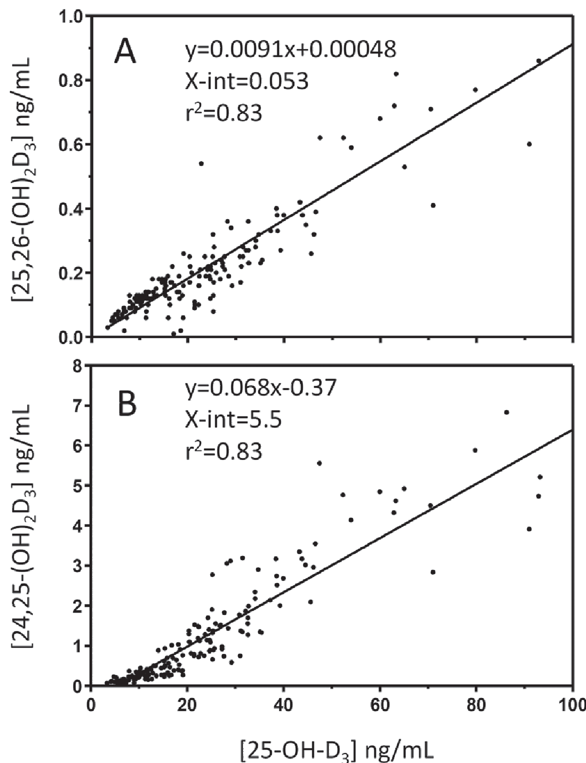

Fig. 5. Relationships between 25-OH-D_3 and its dihydroxylate products over the full normal range of 25-OH-D_3 in a cohort of 156 normal premenopausal and postmenopausal women receiving graded doses of vitamin D_3 for 1 year. (*A*) The relationship between 25-OH-D_3 and 25,26-$(OH)_2D_3$; (*B*) the relationship between 25-OH-D_3 and 24,25-$(OH)_2D_3$.

analysis [CPBA], radioimmunoassay [RIA], gas chromatography–mass spectrometry [GC-MS] mass fragmentographic assay) to measure 25,26-$(OH)_2D_3$ in the past,[19–21] such studies were limited to small numbers of individuals. Our study is a far larger investigation using more accurate LC-MS/MS technology and is the first to study serum 25,26-$(OH)_2D_3$ levels in normals and in two different pathological conditions: IIH and dialysis patients. The serum levels of 25,26-$(OH)_2D_3$ reported here (0.30 to 1.20 ng/mL) compare favorably with those reported earlier.[19–21] With the discovery and focus on the hormone 1,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$ has been largely ignored and assumed to be an inactive catabolite of 25-OH-D_3. Our work does little to dispel the notion of its degradatory role, but it does offer some new in vivo insights into its origins. As with 24,25-$(OH)_2D_3$, its concentration correlates with 25-OH-D_3 over a wide concentration range, suggesting that just as with 24,25-$(OH)_2D_3$, its production at higher concentrations is largely unregulated. Indeed, plots of 25,26-$(OH)_2D_3$ versus 25-OH-D_3 show a similar linear relationship to that of 24,25-$(OH)_2D_3$ versus 25-OH-D_3, except that the former plot appears to have a smaller intercept on the *x* axis, suggesting that 25,26-$(OH)_2D_3$ production remains linear even at very low substrate concentrations.[5,22] The presence of 25,26-$(OH)_2D_3$ in the blood of IIH patients reinforces in vitro work that this metabolite is *not* a product of CYP24A1, although the presence of 25,26-$(OH)_2D_3$ in the blood of dialysis patients is consistent with the finding of others that it is detectable in anephrics[23] and suggesting that it is *not* made in the kidney. Although there are alternative claims that 25,26-$(OH)_2D_3$ is formed in the kidney,[23,24] current dogma suggests that it might be a product of one of the liver enzymes known to metabolize vitamin D, namely CYP2R1, CYP27A1, or CYP3A4. In vivo work with these enzymes will be required to clarify if any one or more of these enzymes are responsible for the synthesis of 25,26-$(OH)_2D_3$.

In conclusion, our work[4,5] has emphasized the importance of the assay of 24,25-$(OH)_2D_3$ in the diagnosis of IIH and other disease states. With this in mind, the pursuit of interlaboratory comparisons will be necessary as 24,25-$(OH)_2D_3$ measurement becomes more widely adopted. Consistent with this, the recent development of a reference method for 24,25-$(OH)_2D_3$ by Tai and Nelson[25] is an important step in this process.

Disclosures

All authors state that they have no conflicts of interest.

Acknowledgments

This work was supported by grants from the National Institute of Standards and Technology (NIST) and NIH-ODS as part of the Vitamin D Standardization Program (VDSP) (Grant 60NANB13D203) and the European Rare Diseases Consortium (E-Rare-2)/Canadian Institutes for Health Research, Grant #ERA-132931) to G Jones. Through a Queen's University/Waters Corporation agreement, Waters Corporation generously provided the LC-MS/MS instrumentation used in these studies. We gratefully acknowledge the provision of individual IIH serum samples from Celia Rodd (University of Manitoba, Winnipeg, MB, Canada), Tom Jacobs (Columbia University, New York, NY, USA), David Saxon (University of Colorado, Denver, CO, USA). We also thank J. Wesley Pike (University of Wisconsin–Madison, Madison, WI, USA) and René St-Arnaud (McGill University and Shriner's Hospital, Montréal, QC, Canada) for the generous gift of serum from *Vdr*-null and *Cyp24a1*-null mice.

Authors' roles: Study design: MK, NM, BJM, DPC, and GJ. Study conduct: MK, NM, BJM, DPC, KPS, AM, MLK, JCG, LA, and GJ. Provision of patient samples and or genetic analysis: KPS, AM, MLK, JCG, and LA. Data collection: MK and NM. Data analysis: MK, NM, and GJ. Data interpretation: MK, NM, BJM, DPC, and GJ. Drafting and revising the manuscript: MK and GJ.

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Pediatric Nephrology (2018) 33:1723–1729
https://doi.org/10.1007/s00467-018-3998-z

ORIGINAL ARTICLE

Prenatal hyperechogenic kidneys in three cases of infantile hypercalcemia associated with *SLC34A1* mutations

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Received: 6 April 2018 / Revised: 21 May 2018 / Accepted: 25 May 2018 / Published online: 29 June 2018
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Abstract

Background Prenatal diagnosis of hyperechogenic kidneys is associated with a wide range of etiologies and prognoses. The recent advances in fetal ultrasound associated with the development of next-generation sequencing for molecular analysis have enlarged the spectrum of etiologies, making antenatal diagnosis a very challenging discipline. Of the various known causes of hyperechogenic fetal kidneys, calcium and phosphate metabolism disorders represent a rare cause. An accurate diagnosis is crucial for providing appropriate genetic counseling and medical follow-up after birth.

Methods We report on three cases of fetal hyperechogenic kidneys corresponding to postnatal diagnosis of nephrocalcinosis. In all cases, antenatal ultrasound showed hyperechogenic kidneys of normal to large size from 22 gestational weeks, with a normal amount of amniotic fluid. Postnatal ultrasound follow-up showed nephrocalcinosis associated with hypercalcemia, hypercalciuria, elevated 1,25$(OH)_2$-vitamin D, and suppressed parathyroid hormone levels.

Results Molecular genetic analysis by next-generation sequencing performed after birth in the three newborns revealed biallelic pathogenic variants in the *SLC34A1* gene, encoding the sodium/phosphate cotransporter type 2 (Npt2a), confirming the diagnosis of infantile hypercalcemia.

Conclusions Nephrocalcinosis due to infantile hypercalcemia can be a cause of fetal hyperechogenic kidneys, which suggests early antenatal anomaly of calcium and phosphate metabolism. This entity should be considered in differential diagnosis. Postnatal follow-up of infants with hyperechogenic kidneys should include evaluation of calcium and phosphate metabolism.

Keywords Antenatal diagnosis · Nephrocalcinosis · Infantile hypercalcemia · *SLC34A1*

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Introduction

Hyperechogenic kidneys without urinary tract anomalies are regularly seen at the time of a routine prenatal ultrasound (US) examination. They represent a difficult diagnosis dilemma, especially when the amniotic fluid volume is normal, since their underlying etiologies are diverse. Hyperechogenicity is the consequence of renal anomalies, such as renal dysplasia, fibrosis, interstitial infiltration, tubular/glomerular dilation, or microcysts. The main etiologies of hyperechogenic kidneys include several diseases encompassing autosomal dominant and recessive polycystic kidney diseases, renal disease associated with *HNF1B* mutations, syndromic ciliopathies (Bardet Biedl and many other ciliary diseases), metabolic diseases, or syndromes including macrosomia such as Wiedemann Beckwith syndrome and others [1]. More rarely, hyperechogenic fetal kidneys are observed in cases of viral infection [2], renal vein thrombosis, congenital nephrotic syndrome, renal tubular dysgenesis, or diseases associated with nephrocalcinosis [3]. Whereas prenatal evaluation of prognosis is mainly based on morphology (extra-renal anomalies, renal size and echogenicity, amniotic fluid volume), establishing an accurate diagnosis is also crucial for providing appropriate genetic counseling and appropriate medical follow-up after birth.

We report on three cases of prenatal hyperechogenic kidneys associated with molecular diagnosis of infantile hypercalcemia (IH) caused by heterozygous biallelic variants in the *SLC34A1* gene.

Patients and methods

Case 1

The mother was seen during pregnancy because fetal US showed hyperechogenic kidneys at 22 weeks of gestation (Fig. 1a). Kidneys were of normal size. Hyperechogenicity was diffuse and corticomedullary differentiation was decreased. Amniotic fluid volume was normal. US performed at 25, 28, 32, and 35 weeks showed similar results (normal-sized hyperechogenic kidneys in a male fetus and normal amniotic fluid volume). Renal US in parents were normal.

A male was born at 38 weeks of amenorrhea, with a birth weight of 2960 g (33rd percentile) and a size of 49 cm (45th percentile). Apgar score was normal. He received breastfeeding and vitamin D 1000 U/day. At 2.5 months of life, he had acute pyelonephritis, and renal US at that time showed typical aspects of nephrocalcinosis (Fig. 2a). Biochemical analyses at that time are shown in Table 1.

Case 2

The second case was referred because of the detection of fetal normal-sized hyperechogenic kidneys during second trimester US examination, with normal amniotic fluid volume, without any other anomalies (Fig. 1b). Her daughter was born at 39 weeks of amenorrhea and 5 days. The birth weight was 3490 g (69th percentile) and the size was 49 cm (41st percentile). A good adaptation to extra-uterine life in the neonatal period was reported. At the time of clinical examination, she was exclusively breast-fed and received vitamin D 1000 U/day. At 1.5 months of life, a renal control ultrasound was performed and showed bilateral pyramidal hyperechogenicity compatible with nephrocalcinosis (Fig. 2b). Biochemical analyses are shown in Table 1. The mother’s serum and urinary analysis 2 months after delivery showed no anomaly; 25 hydroxy-vitamin D was 26 ng/mL.

Case 3

The third pregnant woman was referred after the follow-up US performed at 23 gestational weeks revealed two hyperechogenic kidneys (+ 2SD in size), with no abnormality of corticomedullary differentiation, and normal amniotic fluid volume (Fig. 1c). A girl was born after 41 weeks of gestation,

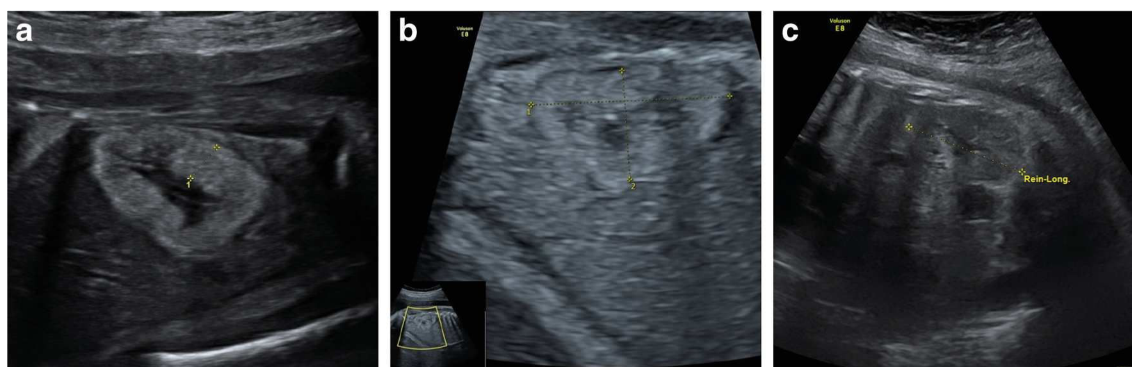

Fig. 1 Prenatal ultrasound (US). **a** Case 1 at 25 weeks of gestation, **b** case 2 at 27 weeks of gestation, and **c** case 3 at 36 weeks of gestation (US pictures at first detection for this case were not available)

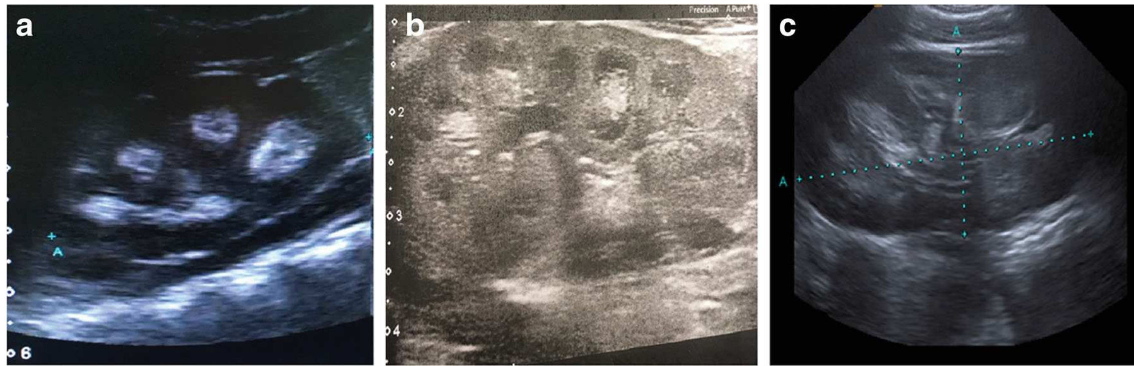

Fig. 2 Postnatal ultrasound. **a** Case 1 at 2.5 months, **b** case 2 at 1.5 months, and **c** case 3 at 3 years

the birth weight was 3220 g (22nd percentile), and the size was 48 cm (7th percentile). Postnatal US at day 4 confirmed hyperechogenic, but well-differentiated, normal-sized kidneys. Biological evaluation at 1.5 months, under breastfeeding and vitamin D 1000 U/day, is shown in Table 1. Vitamin D supplementation was stopped. Serum calcium normalized 3 months later, but hypercalciuria and low plasma parathyroid hormone (PTH) still persisted at 3 years and nephrocalcinosis was stable (Fig. 2c).

Next-generation sequencing

Molecular diagnosis was performed after birth for each case. Genomic DNA was isolated from white blood cells using standard procedures.

For the first two cases, library preparation, massive parallel sequencing, and bioinformatic analysis were performed as in [4]. All of the targeted regions were covered at > 30× except for exon 1 of *OCRL* and *WNK1*. Identified mutations were

Table 1 Biological analysis results of the three infants at the time of diagnosis

	Case 1 [2.5 months]	Case 2 [2 months]	Case 3 [1.5 months]
Plasma [normal range at age]			
Creatinine (μmol L^{-1}) [15–37]	22	28	20
CO_2 total (mmol/L) [19–24]	21.5	23	22
Potassium (mmol/L) [3.1–4.7]	4.6	4.0	5.4
Sodium (mmol/L) [133–140]	137	137	136
Magnesium (mmol/L) [0.6–1.3]	0.75	0.76	NA
Chloride (mmol/L) [95–105]	106	107	103
Total calcium (mmol/L) [2.20–2.83]	*2.68*	*2.72*	*2.97*
Ion calcium (mmol/L) [1.22–1.40]	*1.44*	NA	NA
Phosphate (mmol/L) [1.55–2.39]	*1.68*	*1.72*	*1.7*
PTH (ng/L) [7–31]	5.6	6.9	< 5
25 OH-vitamin D (ng/mL) [50–80]	47	75	130.1
1,25$(OH)_2$-vitamin D (pg/L) [30–150]	*155*	*120*	*188*
Urine			
Calcium/creatinine (mmol/L/mmol/L)	*2.6*	*3.45*	*4.03*
Sodium (mmol/L)	20	< 20	< 10
Phosphate (mmol/L)	3.23	2.76	< 1.62
TRP (%)	–	95.7 [> 85]	> 97
TmP/GFR (mmol/L)	2 [1.48–3.3]	–	> 2.19
pH	7.4	7.4	NA
Genotype			
Allele 1 *SLC34A1**	c.644+1G>A, p.?	c.272_292del, p.Val91_Ala97del	c.272_292del, p.Val91_Ala97del
Allele 2 *SLC34A1**	c.1174G>A, p.Asp392Asn	c.1204G>C, p.Gly402Arg	c.1006+1G>A, p.?

NA not available, *PTH* parathyroid hormone, *GFR* glomerular filtration rate

*Numbering according to the reference cDNA sequence (GenBank: NM_003052.4). Nucleotide 1 is the A of the initiator methionine codon

confirmed by Sanger sequencing. For the third case, library preparation was obtained after multiplex PCR amplification (Ion AmpliSeq On-Demand Panel) (www.thermofisher.com), specifically designed for known genes involved in calcium and phosphorus metabolism. Each library was analyzed on an Ion Torrent Personal Genome Machine (PGM) (Thermo Fisher Scientific, Waltham, MA, USA). Regions with coverage < 30× were sequenced from both strands using Big Dye® Terminator v1.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) on an ABI 3500 Sequencer (Thermo Fisher Scientific, Waltham, MA, USA). Bioinformatics analysis was performed using commercial pipelines (IonSuit, Thermo Fisher Scientific, Waltham, MA, USA, and NextGene, SoftGenetics, State College, PA, USA).

For in silico analysis, we used Alamut V.2.10 software (Interactive Biosoftware, Rouen, France; http://www.interactivebiosoftware.com). Variants were classified according the American College of Molecular Genetics guidelines [5]. All variants identified were confirmed by Sanger sequencing.

Results

For the first case, two heterozygous variants were identified in intron 6 and exon 10 of the *SLC34A1* gene: c.[644+1G>A(;)1174G>A], p.[(?)(;)Asp392Asn] (Fig. 3a). These variants have been previously reported [4, 6] and were classified as classes 5 (pathogenic) and 4 (likely pathogenic), respectively, according to the ACMG guidelines [5]. In addition, the second variant affects the last nucleotide of the exon 10 and might also affect splicing (in silico analysis shows a significant decrease of splicing site scores of 37 and 11.5% for MaxEntScan and SpliceSiteFinder-like programs, respectively) [7]. Parental DNA study showed that the intron 6 variation was inherited from the mother, but neither parent carried the exon 10 variant in blood cell DNA. Non-paternity was excluded by analysis of six unlinked microsatellite markers located on six chromosomes.

For the second case, molecular screening identified two heterozygous variants in exons 4 and 11 of *SLC34A1* (Fig. 3b): c.[272_292del];[1204G>C],

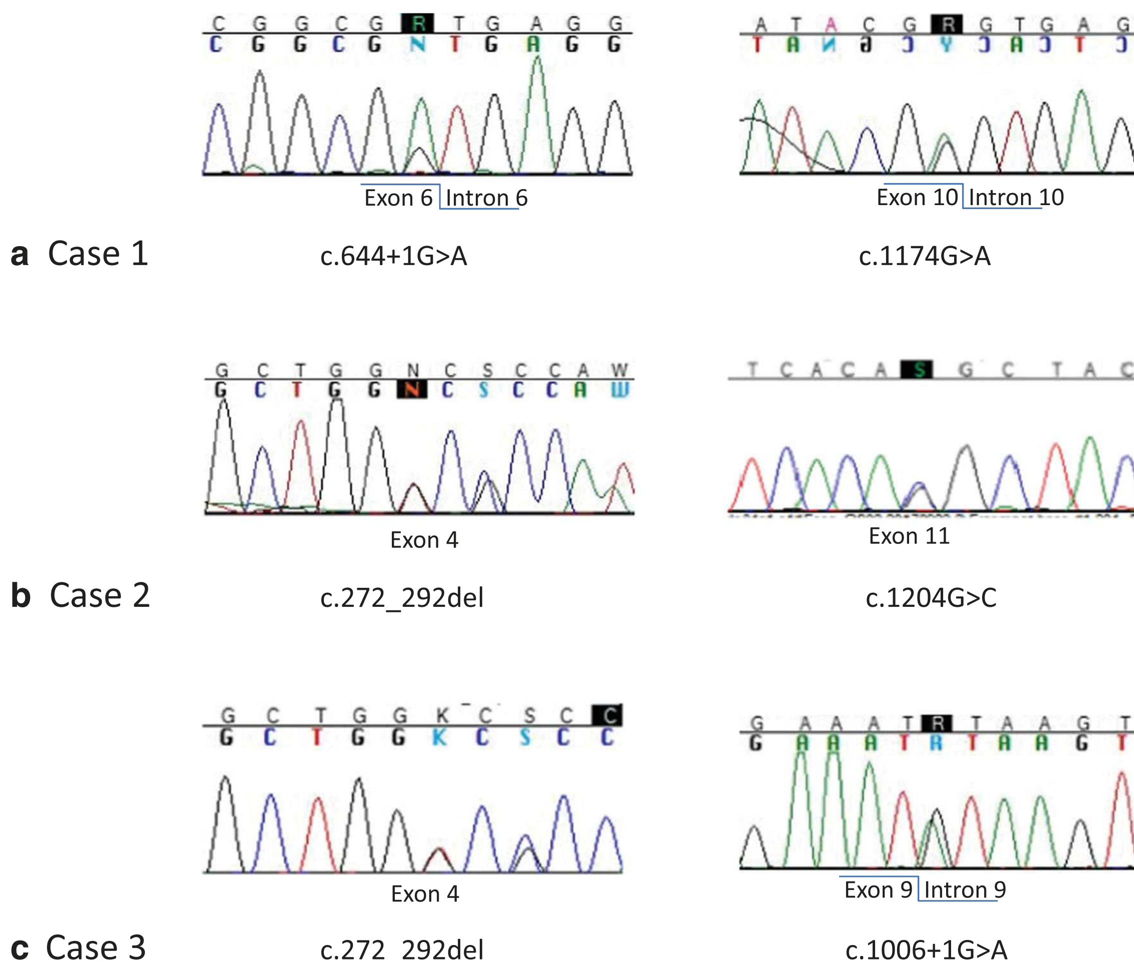

Fig. 3 **a**–**c** Chromatograms of *SLC34A1* mutations detected in the three cases

p.[(Val91_Ala97del)];[(Gly402Arg)]. The first variant is an in-frame deletion of 21 bp that results in the loss of seven amino acid residues and was inherited from the mother. It has been reported previously as not associated with variation in renal phosphate handling in a heterozygous state in adult hypercalciuric stone-former patients, even though heterologous expression in *Xenopus laevis* oocytes demonstrated a reduced expression level [8]. However, another deletion of 21 bp annotated as c.271_291del and having the same consequence at the protein level was described as disease-causing when associated with another pathogenic variant *in trans* or in homozygous status in IH [6]. The second variant, a missense variant in exon 11 inherited from the father, was recently described associated with a splice site mutation in an Egyptian patient affected with nephrocalcinosis [9]. Both variants were classified as class 4 (probably pathogenic) according to the ACMG guidelines [5].

The third case exhibited two heterozygous variants in *SLC34A1* exon 4 and intron 9: c.[272_292del];[1006+1G>A], p.[(Val91_Ala97del);(?)] (Fig. 3c). The first variant, inherited from the mother is the same as described in case 2 and was classified as class 4 according the ACMG guidelines [5]. The second variant, inherited from the father, is a substitution in the essential splicing donor site of intron 9, previously described in [6] and classified as class 5.

Discussion

We report on three cases of fetuses with hyperechogenic kidneys, corresponding to postnatal nephrocalcinosis, indicative of IH associated with mutations of the gene coding for phosphate transporter Npt2a. This gene, located on 5q35.3, encodes a member of the type II sodium-phosphate cotransporter family, expressed in kidney and more specifically on the apical membrane of renal proximal tubule cells. This cotransporter plays a major role in phosphate homeostasis, reabsorbing phosphate from primitive urine, which is regulated by PTH and upregulated by 1,25(OH)2D [10]. Biallelic mutations in *SLC34A1* have been reported in association with Fanconi syndrome [11] (OMIM#613388) and type 2 IH [6] (OMIM #616963). The features presented by our three cases are consistent with IH, which may be caused either by mutations of *SLC34A1* or by mutations of the vitamin D degradation enzyme *CYP24A1* [12]. When associated with *SLC34A1* mutations, the primary renal phosphate wasting caused by defective Npt2a function induces increased production of 1,25(OH)2D, characterized by moderate chronic hypercalcemia associated with hypercalciuria and suppressed PTH levels. In the previously described cases of IH (associated with *CYP24A1* or *SLC34A1* mutations) [12] [6, 12–18], only one case of prenatal renal hyperechogenicity was reported in a male patient with genetic diagnosis of IH at adolescence (homozygous mutation of *SLC34A1*) and medullary nephrocalcinosis on prenatal ultrasound [13].

In physiological conditions, fetal levels of calcium and phosphate are maintained higher than the maternal levels to meet the requirements of the developing fetus, thanks to placental active transport in the maternofetal direction. $1{,}25(OH)_2D$ level is lower in the fetus than in the mother, and the production of $1{,}25(OH)_2D$ does not seem to be under the control of the classic regulators of calcium, phosphorus, and/or PTH during pregnancy. Levels of calcium and phosphorus are mainly regulated in the fetus by PTHrp, and phosphate level is maintained within the normal range despite maternal hypophosphatemia [19–21].

The presence of antenatal hyperechogenic kidneys suggests prenatal nephrocalcinosis associated with a dysregulation of fetal calcium and phosphate metabolism, although we cannot exclude prenatal renal hyperechogenicity of another cause. Compared with the calcium transport system, the mechanism for transplacental transport of Pi is largely unknown. It is suggested that the placental transfer of Pi in the maternofetal direction also occurs in a transcellular manner and depends on an Na+- and pH-dependent active transport mechanism [19]. However, this seems to mainly involve another phosphate transporter Npt2b, which is predominantly expressed in the intestine in postnatal life [22]. Placental expression of *SLC34A1*, encoding Npt2a, was shown to be increased in late gestation, although still very low [19], and thus, this transporter is unlikely to have a significant role in transplacental Pi transport.

In our three cases, the diagnosis of nephrocalcinosis after birth suggests that the prenatal renal hyperechogenicity was due to calcium deposits during fetal life. We can speculate that the prenatal pathophysiology of hypercalciuria is the same as in patients with IH, i.e., transplacental transport cannot compensate the urinary loss of Pi, with the development of fetal hypophosphatemia and hypercalcitriolemia (the fetal kidney expresses 1α-hydroxylase) [20]. However, antenatal nephrocalcinosis has not been described in fetuses with other diseases with abnormal phosphate reabsorption, such as hereditary hypophosphatemic rickets with hypercalciuria (HHRH). In any case, because we do not know the calcium, phosphorus, and vitamin D levels in the mothers during pregnancy, it is difficult to discuss the role of the maternal environment. Along the same lines, it would have been interesting to measure calcium, phosphorus, and vitamin D in amniotic fluid.

Nephrocalcinosis results from an imbalance between factors promoting kidney calcium deposits (hypercalciuria) and factors inhibiting them, such as osteopontin. Interestingly, a decrease of renal gene expression and urinary excretion of osteopontin has been shown in *Npt2a*$^{-/-}$ mice, which may also play a role in the development of nephrocalcinosis [23]. Further studies will be necessary to (1) understand the

prevalence of renal antenatal abnormalities in IH, (2) investigate if they are exclusively associated with *SLC34A1* mutation or could also be observed in association with mutations in *CYP24A1*, (3) elucidate the pathophysiology of prenatal nephrocalcinosis, and (4) determine whether calcium deposits always begin in the prenatal period, or if there are additional factors that determine the age of onset and severity of nephrocalcinosis.

Few data are available for renal function follow-up in IH patients with genetic confirmation. Among available data in patients older than 18 years, chronic kidney disease (CKD) stages III and IV has been described in 4 patients (at 24, 47, 35, and 46 years old) [13, 15, 18]. Pronicka et al. recently reported genetic confirmation in historical cases of IH; they showed absence of CKD in 11 patients at mean age of 27 years [16]. Nevertheless, 2 cases presented with a borderline creatinine, corresponding to stage II K-DOQI. We do not know if other factors in addition to nephrocalcinosis had contributed to CKD in these patients. In any case, monitoring and appropriate treatment (avoiding excessive vitamin D and calcium dietary intake and risky behaviors such us long sunlight exposures) can help to prevent the development of stones and nephrocalcinosis and finally to preserve kidney function in IH patients.

In conclusion, nephrocalcinosis due to IH can be a cause of fetal hyperechogenic kidneys and should be considered as part of the differential diagnosis. Postnatal follow-up of infants with hyperechogenic kidneys should include evaluation of calcium and phosphate metabolism. Further studies are necessary to better understand the antenatal pathophysiology and the long-term consequences on renal function of IH.

Funding information Funding for this study was in part provided by the European Union, FP7 (grant agreement 2012-305608) “European Consortium for High-Throughput Research in Rare Kidney Diseases (EURenOmics).”

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Case Report
AJKD

Fluconazole as a New Therapeutic Tool to Manage Patients With NPTIIc (*SLC34A3*) Mutation: A Case Report

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Mutations in the *SLC34A3* gene, encoding the sodium/phosphate cotransporter 2C (NPTIIc), induce decreased renal phosphate reabsorption, hypophosphatemia, decreased fibroblast growth factor 23 and parathyroid hormone, and increased 1,25-dihydroxyvitamin D (1,25[OH]$_2$D) levels. The complete phenotype is characterized by hypophosphatemia, hypercalciuria, and nephrolithiasis/nephrocalcinosis, leading to chronic kidney disease and osteoporosis in adults. We report a 15-year-old boy referred for nephrocalcinosis. The patient demonstrated hypercalcemia, hypercalciuria, normal serum phosphate level, normal tubular phosphate reabsorption, and increased serum 1,25(OH)$_2$D level with suppressed serum parathyroid hormone. Compound heterozygous mutations in *SLC34A3* were found. Hydrochlorothiazide failed to decrease calciuria. Fluconazole, an inhibitor of 1α-hydroxylase, was effective in normalizing calciuria without decreasing glomerular filtration rate. We conclude that children with *SLC334A3* mutations can present with a less-typical phenotype, having normal serum phosphate levels and normal renal phosphate reabsorption. Genetic abnormalities of NPTIIc should be considered in cases of increased 1,25(OH)$_2$D levels without mutations in *CYP24A1*. The utility of fluconazole to decrease 1,25(OH)$_2$D levels requires confirmation in larger studies.

Complete author and article information provided before references.

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Am J Kidney Dis. XX(XX): 1-4. Published online Month X, XXXX.

doi: 10.1053/j.ajkd.2018.12.026

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Introduction

Hypercalciuria is one of the most frequent metabolic disorders associated with nephrolithiasis and/or nephrocalcinosis. Due to the availability of genetic testing and improvement in genetic knowledge, some cases of "idiopathic" hypercalciuria can now be explained by mutations in *CYP24A1* (encoding the vitamin D 24-hydroxylase enzyme) or *SLC34A1*/*SLC34A3*.[1,2] These latter genes encode the sodium/phosphate cotransporters 2A and 2C (NPTIIa and NPTII2c), respectively, which are located in the apical membrane of renal proximal tubular cells and responsible for phosphate reabsorption.[3,4] Mutations in these genes are associated with renal phosphate wasting, as expressed by decreased maximum tubular reabsorption of phosphate normalized for glomerular filtration rate (TmP/GFR). This impairment in renal phosphate reabsorption usually induces hypophosphatemia, further leading to downregulation of fibroblast growth factor 23 (FGF-23) and parathyroid hormone, but also to increased 1,25-dihydroxyvitamin D (1,25[OH]$_2$D) levels. The latter stimulates intestinal phosphate and calcium absorption and promotes hypercalciuria. Thus, the complete phenotype is characterized by hypophosphatemia, hypercalciuria, and nephrolithiasis/nephrocalcinosis. Chronic kidney disease (CKD) and osteoporosis may eventually result. Less severe and/or atypical phenotypes have also been described, notably in patients with heterozygous or compound heterozygous mutations: in such patients, hypophosphatemia is not consistently reported.[2]

Management of these genetic causes of hypercalciuria usually consists of hyperhydration and dietary advice for a low-sodium diet with normal calcium intake. In cases of overt hypophosphatemia and subsequent abnormal bone mineralization (as defined by increased alkaline phosphatase levels), phosphate supplementation may be prescribed during childhood, similar to the treatment of X-linked hypophosphatemic rickets. However, the proper approach to this condition remains uncertain because there are no definitive studies available. The addition of hydrochlorothiazide can be considered to reduce hypercalciuria but requires close follow-up of serum calcium and potassium levels, as well as monitoring for other side effects.[5] The utility of ketoconazole has been reported in patients with increased 1,25(OH)$_2$D levels in those with loss-of-function mutations in *CYP24A1* or *FGF23*.[6,7] However, its long-term off-label use may be problematic, mainly because of its potential liver toxicity.[8] Other authors have reported the successful use of fluconazole, another azole drug that may be less toxic, in patients with *CYP24A1* mutation.[9]

We report a young patient with *SLC34A3* mutation with an incomplete phenotype in whom fluconazole therapy corrected hypercalciuria and normalized 1,25(OH)$_2$D and 25-hydroxyvitamin D levels.

Case Report

A 15-year-old boy without family or personal medical history was referred because of the incidental diagnosis of renal calcifications on x-rays obtained to investigate scoliosis. Renal ultrasound showed medullar nephrocalcinosis without nephrolithiasis. Growth was normal and neither bone deformation nor bone pain was reported. As illustrated in Table 1, laboratory assessment revealed hypercalcemia, hypercalciuria, low parathyroid hormone level, 25-hydroxyvitamin D concentration within the reference range, and increased 1,25(OH)$_2$D level. Serum phosphate level and TmP/GFR were within the reference ranges for age. Proteinuria was absent and there was no

Table 1. Evaluation of Biochemical Values Before and After Fluconazole Treatment

	Reference Range[a]	Before Any Treatment	On Hydrochlorothiazide, 25 mg/d	On Fluconazole	
				50 mg/d	100 mg/d
Serum calcium, mmol/L	2.1-2.55	2.72	2.63	2.54	2.39
Ionized calcium, mmol/L	1.1-1.3	1.4	1.35	1.22	ND
Phosphate, mmol/L	0.94-1.62	1.25	1.17	ND	0.87
25-Hydroxyvitamin D_3, nmol/L	50-125	97	72	78	64
1,25-Dihydroxyvitamin D, pmol/L	69-200	279	387	151	95
PTH, ng/L	15-65	8	7	6	16
Urinary calcium, mmol/kg/d	<0.1	0.19	0.15	0.14	0.08
TmP/GFR, mmol/L	0.75-1.35	1	ND	ND	0.7
Serum creatinine, μmol/L		85	87	ND	119
mGFR, mL/min/1.73 m²	>90	69	ND	ND	64
Renal ultrasound		Bilateral medullary nephrocalcinosis			Bilateral medullary nephrocalcinosis (stable)

Abbreviations: mGFR, measured glomerular filtration rate (using iohexol clearance); ND, not determined; PTH, parathyroid hormone; TmP/GFR, maximum tubular reabsorption of phosphate normalized for glomerular filtration rate.
[a]Local values from individuals of comparable age.

evidence for a generalized proximal tubulopathy. GFR was decreased to a level corresponding to CKD stage 2. Sarcoidosis was ruled out; genetic testing did not demonstrate an abnormality in *CYP24A1* but revealed compound heterozygous mutations in *SLC34A3* (c.925+20_926-48del [a deletion in intron 9 that leads to abnormal splicing] and c.1055_1058dup [a duplication in exon 10 predicted to lead to a frameshift and premature termination of the protein]; Fig S1).

Hydrochlorothiazide was prescribed for 2 years (25 mg/d) with no effect on urinary calcium excretion. At the age of 17 years, because of persistent hypercalciuria and increased $1,25(OH)_2D$ levels, fluconazole was introduced at a daily dose of 50 mg and increased up to 100 mg per day 9 months later. This led to normalization of urinary calcium excretion, as illustrated in Table 1. Liver enzyme levels were closely monitored and remained normal. At the age of 18.5 years, GFR was stable and plasma and urinary calcium levels were normal; bone density, assessed using dual x-ray absorptiometry, was decreased with a lumbar T score of −2.6 and femoral T score of −2.7.

This retrospective review of medical charts was approved by the local institutional review board (Comité d'Ethique des Hospices Civils de Lyon, session June 7, 2018), and the patient approved the publication of this observation.

Discussion

The existence of nephrolithiasis and/or nephrocalcinosis in children or young adults requires a complete evaluation of the components of calcium and phosphate metabolism. Hypercalciuria and increased $1,25(OH)_2D$ levels with normal phosphate metabolism are usually due to primary abnormalities in vitamin D metabolism. However, *CYP24A1* mutations explain only 35% of these cases.[10] Genes not yet identified in vitamin D metabolism may explain some of the cases. However, we also show here that *SLC34A3* mutations can be found in these less-typical clinical situations despite normal serum phosphate levels and normal tubular reabsorption of phosphate corrected for the patient's age. One may argue that because our patient had CKD stage 2, this could have resulted in normalization of serum phosphorus levels. However, in the CKiD (CKD in Children) cohort, it was shown that children with CKD stage 2 typically display normal to low serum phosphorus levels.[11]

We identified 2 mutations in *SLC34A3* in a compound heterozygous state that are considered to be pathogenic.[12] The first was a maternally inherited intronic 101–base pair deletion responsible for abnormal splicing (retention of the truncated intron 9 in the messenger RNA) that was previously published in patients with hereditary hypophosphatemic rickets with hypercalciuria.[13] The second was a paternally inherited 4–base pair deletion in exon 10, which, although it was absent from a database of mutations and polymorphisms, is predicted by pathogenicity algorithms to lead to a frameshift.

Although they have different genetic bases, some diseases share inappropriately increased $1,25(OH)_2D$ levels as a direct or indirect result of the underlying genetic defect. This is the case in hypersensitivity to vitamin D (*CYP24A1* mutations, or neonatal severe hypercalcemia without genetic explanation), familial tumoral calcinosis (low active FGF-23 levels), and hypercalciuria with renal phosphate wasting (mutations in the genes encoding NPTIIa, NPTIIc, and NHERF1 [sodium/hydrogen exchanger regulatory factor 1]), as illustrated in Figure 1.[3,7,10,14,15]

The use of ketoconazole and fluconazole has been reported in some of these genetic diseases.[7,9,14] These azoles effectively inhibit the vitamin D 25- and 1α-hydroxylases and therefore decrease $1,25(OH)_2D$ levels. In this case of an *SLC34A3* mutation, hypercalciuria normalized while GFR remained stable, and no other side effects were reported during follow-up.

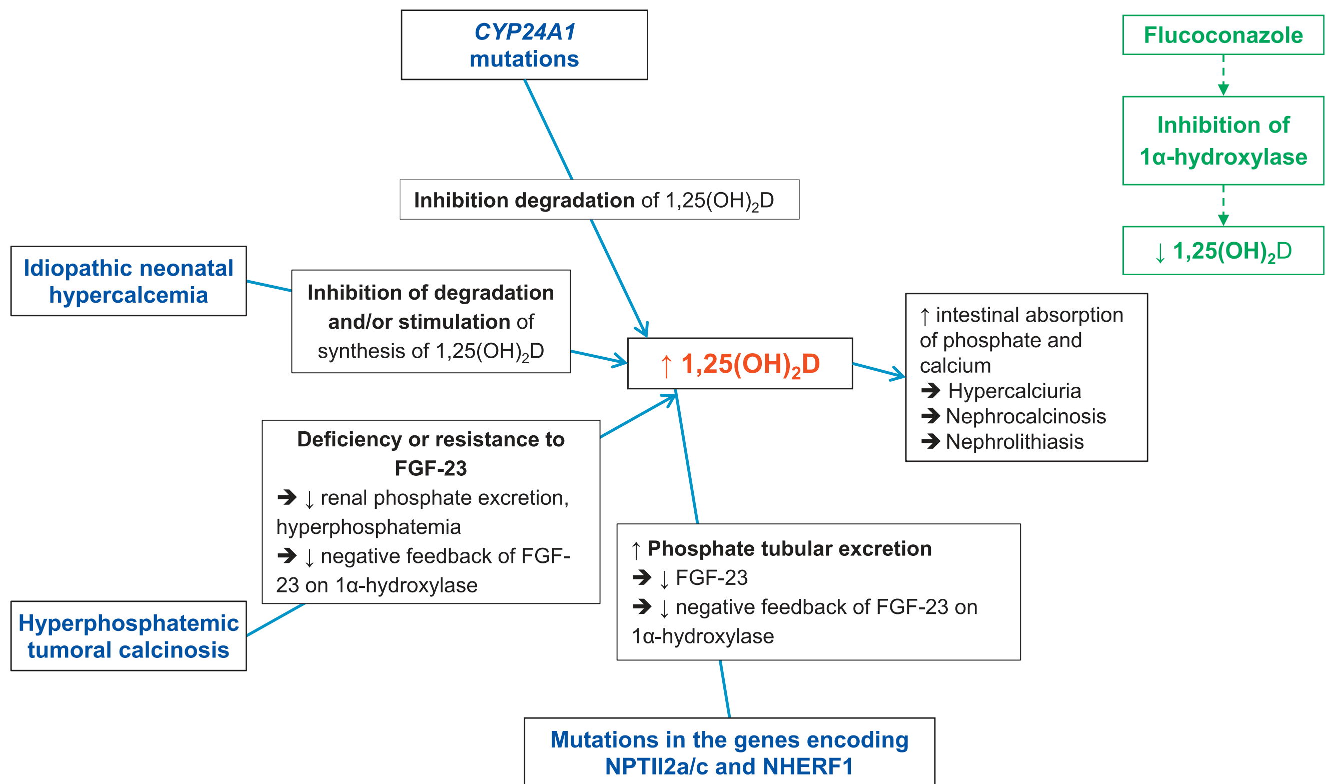

Figure 1. Putative mechanisms of action of fluconazole in hypercalciuria with increased 1,25-dihydroxyvitamin D (1,25[OH]$_2$D) levels. Fibroblast growth factor 23 (FGF-23) inhibits 1,25(OH)$_2$D through at least 2 direct mechanisms: inhibition of 1α-hydroxylase and stimulation of 24-hydroxylase, thus leading to decreased 1,25(OH)$_2$D levels. In contrast, 1,25(OH)$_2$D is stimulated by (absolute or relative) hypocalcemia, (absolute or relative) hypophosphatemia, and parathyroid hormone. Although they have a different pathophysiology, some diseases share inappropriately increased 1,25(OH)$_2$D levels as a direct or indirect result of the underlying defect. This is the case, for example, for hypersensitivity to vitamin D (*CYP24A1* mutations, or neonatal severe hypercalcemia without genetic explanation), familial tumoral calcinosis (low active FGF-23 levels), and hypercalciuria with renal phosphate wasting (mutations in the genes encoding sodium/phosphate cotransporters 2A and 2C [NPTIIa and NPTIIc] and sodium/hydrogen exchanger regulatory factor 1 [NHERF1]). In patients with mutations in the genes encoding NPTIIa or NPTIIc, the decreased tubular phosphate reabsorption (because of the loss of function of NPTIIa/c) induces both "downregulation" of FGF-23 (which in turn decreases the inhibition of 1,25(OH)$_2$D by FGF-23) and direct stimulation of 1,25(OH)$_2$D to increase intestinal phosphate absorption to maintain circulating phosphate levels, even in the absence of overt hypophosphatemia. Thus, these 2 pathways explain the increased 1,25(OH)$_2$D levels observed in these patients. The use of ketoconazole and fluconazole has already been reported in some of these genetic diseases: these azoles are effective to inhibit 1α-hydroxylase and therefore decrease 1,25(OH)$_2$D levels.

However, the off-label prescription of azoles to decrease 1,25(OH)$_2$D levels should be done with caution. Ketoconazole is known to potentially induce severe side effects, such as hepatic toxicity, prolonged QT interval, and adrenal insufficiency. Fluconazole may be less toxic and Sayers et al[9] used low-dose fluconazole (50 mg/d) in a patient with *CYP24A1* mutation over 98 days to normalize hypercalciuria and reduce 1,25(OH)$_2$D levels from 307 to 179 pg/mL. Given our patient's high 1,25(OH)$_2$D levels at initiation of therapy (ie, 387 pg/mL), we administered larger fluconazole doses than previously reported, anticipating that higher doses would be needed to normalize the hypercalciuria. It is worth noting that compared with typical anti-infectious doses of fluconazole (200-400 mg in adults and 3-12 mg/kg in children, and up to 800-1,200 mg for specific indications),[16] the doses used here are relatively low.

When using fluconazole in patients with *SLC34A3* mutations, there are theoretical risks. First, fluconazole, by decreasing 1,25(OH)$_2$D levels, may induce or worsen hypophosphatemia by reducing gastrointestinal phosphate absorption and/or stimulating parathyroid hormone release. However, after more than 2 years of treatment, our patient did not develop hypophosphatemia. Second, oral phosphate supplementation, if not properly monitored, may worsen nephrocalcinosis. As such, we would suggest that management of this condition be performed in centers with experience in the management of similar conditions, with close monitoring of both phosphate and alkaline phosphatase levels.

Last, in our patient, the decrease in urinary calcium excretion during fluconazole treatment appears to be dose dependent. This observation could provide a rationale to

progressively increase fluconazole doses during follow-up depending on the initial clinical response. Increased fluid intake and possibly the concurrent use of oral citrate therapy may be useful to ameliorate hypercalciuria.

In conclusion, we propose 3 main points emanating from this case. First, *SLC34A3* mutations can be found in patients with normal phosphate levels and normal phosphate tubular reabsorption. Second, genetic analyses of *SLC34A3* may be useful in cases of hypercalciuria and nephrocalcinosis with increased $1,25(OH)_2D$ levels without *CYP24A1* mutations. Last, we provide proof of concept for the use of fluconazole to control increased $1,25(OH)_2D$ levels in the context of *SLC34A3* mutations. The latter requires confirmation in larger prospective studies.

Supplementary Material

Figure S1: Genetic analysis of *SLC34A3*.

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Support: None.

Financial Disclosure: The authors declare that they have no relevant financial interests.

Acknowledgements: We thank Dr Marie-Christine Carlier (PharmD, Hospices Civils de Lyon) for help for biochemical evaluation of the patient.

Peer Review: Received August 2, 2018. Evaluated by 2 external peer reviewers, with direct editorial input from the Education Editor and a Deputy Editor. Accepted in revised form December 6, 2018.

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<u>Etude des causes génétiques de dérégulation du métabolisme de la vitamine D</u>

Résumé :
La vitamine D (D_3 ou cholécalciférol du règne animal et D_2 ou ergostérol du règne végétal) est une hormone pléiotrope qui possède de nombreux effets biologiques incluant la régulation du métabolisme du calcium et du phosphate. Chez l'Homme, ce composé est synthétisé au niveau cutané sous forme inactive. On décrit ainsi le métabolisme de la vitamine D qui conduit à la production de métabolites actifs (par les vitamine D 25- et 1α-hydroxylases codées par les gènes *CYP2R1* et *CYP27B1*) et à leur dégradation par la vitamine D 24-hydroxylase (gène *CYP24A1*). L'expression des vitamine D 1α- et 24-hydroxylases est finement et inversement régulée afin de maintenir l'homéostasie phosphocalcique, grâce à plusieurs boucles de rétrocontrôle impliquant entre autres la forme 1,25-dihydroxylée de la vitamine D et son récepteur VDR, la calcémie et la parathormone, la phosphatémie et le FGF23. La carence en vitamine D et les défauts de son activation sont associés à un phénotype de rachitisme, tandis que les excès en vitamine D sont associés à un phénotype d'hypercalcémie-hypercalciurie par intoxication (surdosage) ou hypersensibilité à la vitamine D (excès d'activation ou défaut de dégradation).
L'objectif de ce travail de thèse est d'identifier des causes génétiques de dérégulation du métabolisme de la vitamine D et de préciser leurs mécanismes physiopathologiques par une description précise du phénotype associé. Pour ce faire, nous avons utilisé de façon conjointe les outils de la génétique (séquençage nouvelle génération et Sanger) et de la biochimie (dosage des métabolites) dans une cohorte de patients recrutés grâce au centre de référence maladies rares du métabolisme du calcium et du phosphate.
Ce travail a permis de préciser le rôle de deux gènes dans les maladies liées à la dérégulation métabolisme de la vitamine D, *CYP2R1* et *CYP24A1*, par la mise en évidence de mutations perte de fonction chez des patients avec un phénotype de rachitisme à 25-hydroxyvitamine D basse et d'hypersensibilité à la vitamine D respectivement. Notre étude a permis aussi de préciser le phénotype de ces affections. Dans la cohorte des patients étudiés, l'identification de mutations de gènes impactant le métabolisme du phosphate (*SLC34A1* et *SLC34A3*), souligne l'intérêt de l'étude des facteurs régulateurs des activités vitamine D 1α- et 24-hydroxylases.
Aucune variation significative dans les régions promotrices proximales de *CYP27B1* et *CYP24A1* n'a été identifiée. Le peu de connaissances sur l'ensemble des éléments régulateurs chez l'Homme n'a pas permis d'approfondir notre étude. L'identification et l'étude de ces éléments régulateurs distaux permettra de déterminer leur implication dans les maladies rares du métabolisme de la vitamine D.

Abstract :
The vitamin D (D_3 or cholecalciferol from animal kingdom and D_2 or ergosterol from plan kingdom) is a pleiotropic hormone who has numerous biological effects including the regulation of calcium and phosphate metabolism. In humans, this compound is synthetized in skin in an inactive form. Thus, we call vitamin D metabolism the biological process which leads to the production of active metabolites (by enzymes 25- and 1α-hydroxylases encoded by *CYP2R1* and *CYP27B1* genes) and its degradation by vitamin D 24-hydroxylase (gene *CYP24A1*). The expression of 1α- and 24-hydroxylases is tightly and inversely regulated to maintain calcium and phosphate homeostasis, thanks to several feedback loops including 1,25-dihydroxyvitamin D and its receptor VDR, serum calcium and parathormone, serum phosphate and FGF23. Vitamin D deficiency and vitamin D activation deficiency are associated with rickets, while vitamin D excess are associated with hypercalcemia-hypercalciuria due to vitamin D intoxication (overdose) or hypersensitivity to vitamin D (activation excess or degradation deficiency).
Our aim is to identify genetic causes of vitamin D metabolism deregulation and to specify pathophysiologic mechanisms describing phenotype. Thus, we jointly used the tools of genetics (next-generation and Sanger sequencing) and biochemistry (vitamin D metabolites assay) in a cohort of human patients ascertained thanks to the national center for rare diseases of calcium and phosphate metabolism.
This work allowed us to specify the role of two genes in diseases of vitamin D metabolism, *CYP2R1* and *CYP24A1*, showing loss of function mutations in patients with rickets and low 25-hydroxyvitamin D and hypersensitivity to vitamin D, respectively. Our study brought new phenotypic elements in these affections. In our cohort of patients, the identification of mutations leading to phosphate deregulation (in *SLC34A1* and *SLC34A3*) highlights the putative role of regulators of vitamin D 1α- and 24-hydroxylases activities in pathophysiology.
No significant variation have been identified in the proximal promoting regions of *CYP27B1* and *CYP24A1*. We could not go further considering the lack of knowledge in regulating regions and factors in humans. Identifying distal regulators will allow to study their implication in rare diseases of vitamin D metabolism.

Mots clefs : vitamine D – génétique – hypercalcémie – rachitisme

Key words : vitamin D – Genetics – hypercalcemia - rickets