BRUNA DA CONCEIÇÃO CORREIA PARREIRA

Genetic variants associated with ectopic calcifications

Doutoramento em Ciências Biotecnológicas

(Especialidade em Biotecnologia Molecular)

Trabalho efetuado sob a orientação de:

Doutor Jácome Bruges Armas

Doutora Ana Rita Couto Rendeiro

Professora Doutora Deborah Power

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2017

Genetic variants associated with ectopic calcifications

Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

(Bruna da Conceição Correia Parreira)

O trabalho aqui apresentado foi realizado no Serviço Especializado em Epidemiologia e Biologia Molecular (SEEBMO) do Hospital de Santo Espirito de Angra do Heroísmo, liderado pelo Doutor Jácome Bruges Armas e no Centro de Ciências do Mar (CCMAR) da Universidade do Algarve, liderado pelo Professor Doutor Adelino Canário.

O trabalho foi co-financiado pelo Fundo Regional da Ciência e Tecnologia dos Açores através do projeto M3.1.2/F/023/2011 e pela Fundação para a Ciência e Tecnologia (FCT-Portugal) através do projeto UID/Multi/04326/2013.

AGRADECIMENTOS

Abro esta dissertação de doutoramento agradecendo a todos aqueles que de forma direta ou indireta estiveram comigo ao longo de todo este percurso e que sem os quais não se teria tornado numa realidade. Agora, que alcanço os meus objetivos não poderia deixar de reconhece-las.

No plano institucional, agradeço à Direcção Regional da Ciência e Tecnologia pela concessão de uma bolsa de doutoramento (M3.1.2/F/023/2011), e ao Hospital de Santo Espirito da Ilha Terceira e ao SEEBMO, pelo apoio ao meu projeto e pelas excelentes condições de acolhimento que me proporcionaram para o desenvolvimento desta investigação. Muito obrigada, pois sem o vosso apoio este doutoramento não teria sido viável. Quero ainda agradecer à Junta de Freguesia dos Altares pelo apoio na impressão desta tese.

Ao meu orientador Doutor Jácome Bruges Armas por me ter colocado o desafio de fazer um doutoramento, pela competência científica e pela orientação dada com toda a dedicação e compreensão. Para mim, foi uma imensa honra e orgulho tê-lo como orientador por mais de 6 anos (desde o mestrado).

A minha profunda gratidão à minha co-orientadora Doutora Ana Rita Couto Rendeiro, pelos seus preciosos conselhos e discussões de ideias que em muito me ajudaram a percorrer este caminho. Obrigada pela sua amizade, por todas as palavras de encorajamento e acima de tudo por ter sempre acreditado em mim.

À minha co-orientadora Doutora Deborah Mary Power da Universidade do Algarve pela sua orientação sempre sábia, generosa e paciente relativamente às duvidas que me iam surgindo e aos problemas que tive de enfrentar. Apesar do Atlântico nos separar a sua voz do outro lado do telefone foi sempre jovial e encorajadora.

À equipa do CCMAR, a qual tive a oportunidade de conhecer graças a uma temporada de um mês que passei na Universidade do Algarve. Quero agradecer especialmente à Doutora Rute Felix e ao meu amigo Doutor João Cardoso que tanto admiro pela forma humilde e paciente como compartilha o saber. Muito obrigada pelo convite e principalmente por todos os ensinamentos dados. Um agradecimento também à Rita Costa pelo trabalho de expressão nos peixes.

A todos os colegas e amigos do SEEBMO, com quem convivi diariamente, partilhando receios e ambições e dos quais sempre tive o maior apoio. Em particular, quero agradecer à Marta, Fabiana, Bruno e Vanessa por toda a colaboração que tiveram neste trabalho.

Um agradecimento muito especial à Madalena que para além de ter colaborado neste trabalho me ajudou imenso na formatação desta tese. Também um agradecimento especial à Fernanda e Diana pela ajuda na recolha da informação das amostras e à enfermeira Vânia do HSEIT pela ajuda na elaboração das árvores das famílias.

A toda a minha família, que seguramente são os que mais compartilham da minha alegria; aos meus queridos pais que sempre estiveram do meu lado me incentivando e que não mediram esforços para que eu chegasse até ao fim desta etapa. A todos os meus irmãos pelos momentos de alegria e distração, em particular à minha querida irmã Cidália que se mostrou sempre disponível para me ajudar em qualquer coisa que precisasse. Aos meus sogros, por amarem e protegerem o meu filho quando não pude estar presente e pela sua generosa ajuda. Ao meu marido Rui pelo seu companheirismo e acima de tudo por ter muitas vezes acolhido as minhas inseguranças e as ter revertido em força. E por ultimo, ao meu bem mais precioso, ao meu filho Lucas, a quem dedico este trabalho; pelo seu lindo sorriso, pela sua ternura, enfim… pelo simples facto de existir. Foram muitas horas que lhe roubei, mas espero poder lhe retribuir este tempo que não vivemos, embora esteja certa que a minha valorização pessoal terá um contributo para a sua educação.

E por fim, agradeço a Deus por me ter conduzido até aqui e por nunca me ter faltado!

Mais uma vez, a todos os meus sinceros agradecimentos.

ABSTRACT

Diffuse idiopathic skeletal hyperostosis (DISH) is a common skeletal disorder characterized by the presence of new bone formation in ligaments and entheses. DISH can co-exist with Chondrocalcinosis (CC) and it has been suggested that both diseases share the same pathogenic mechanism. To date, two genes, *COL6A1* and *FGF2*, have been shown to have a weak positive association with DISH susceptibility. The main objective of this thesis was to investigate the genetic basis of the DISH/CC disease, making use of Next Generation Sequencing technology, association and expression studies, in a group of DISH/CC samples from the Azores biobank. Two regulatory variants in the *RSPO4* gene were significantly more frequent in controls than in DISH/CC patients. These may protect against the DISH/CC phenotype, possibly by altering gene expression of the *RSPO4* gene. Using whole exome sequencing we identified a significant association between the DISH/CC disease and a genetic variant in *BMP4* (rs17563), a gene involved in endochondral bone formation. Another of the candidate genes associated with DISH/CC was *ABCC6* that is of relevance in ectopic calcification disorders. Although inconclusive, the expression studies performed in human cartilage tissue indicated overexpression of *ABCC6* in DISH and CC patients relative to the controls, raising the hypothesis that this gene may be involved in calcium pyrophosphate formation in DISH and CC. A comparative approach using teleosts revealed that the *abcc6* gene is expressed in skin but was not associated with ectopic calcification of the scales. Furthermore, comparative genomics revealed the *abcc6* has only been retained in the genome of bony vertebrates. In summary, I identify for the first time potential gene variants that protect (*RSPO4*) or predispose (*BMP4*) to DISH/CC. The relevance of the *ABCC6* gene in this phenotype remains to be proven. It is unlikely that one major gene is responsible for DISH/CC and instead it appears to be a polygenic disease.

Keywords: Chondrocalcinosis, DISH, WES, *BMP4*, *ABCC6*, *RSPO4*.

RESUMO

A hiperostose idiopática difusa do esqueleto (DISH) é uma doença musculoesquelética comum caracterizada pela formação óssea de novo em ligamentos e enteses. A DISH pode coexistir com a condrocalcinose e por isso tem sido sugerido que ambas partilham o mesmo mecanismo patogénico. *COL6A1* e *FGF2* são os dois genes de suscetibilidade conhecidos com uma ligação genética fraca à DISH.

O objetivo principal desta tese foi investigar a genética da DISH/CC, utilizando a sequenciação de nova geração e estudos de associação e expressão, num grupo de amostras de doentes com DISH/CC do AZORBIO.

Duas variantes na região reguladora do gene *RSPO4* são significativamente mais frequentes nos controlos do que nos doentes DISH/CC. Estas variantes podem afetar a expressão do gene, conferindo proteção à doença. Utilizando a sequenciação exómica identificamos uma associação significativa entre a DISH/CC e a variante genética rs17563 no gene *BMP4*, um gene diretamente envolvido na formação óssea endocondral.

Outro gene candidato estudado foi o *ABCC6*, que parece ser relevante em doenças caracterizadas por calcificações ectópicas. Embora inconclusivos, os estudos de expressão em tecidos de cartilagem humana mostraram que o gene *ABCC6* apresenta expressão superior nos doentes DISH e CC em relação a um doente controlo, levantando a hipótese de que este aumento de expressão poderá estar envolvido com a deposição de cristais de pirofosfato de cálcio nestes doentes. Uma abordagem comparativa utilizando teleósteos revelou que o gene *abcc6* está expresso na pele dos peixes, mas não está associado com a calcificação ectópica das escamas. Além disso, nos resultados da genómica comparativa o gene *abcc6* só foi encontrado no genoma de vertebrados ósseos, indicando que este gene poderá estar envolvido em inovações específicas dos vertebrados.

Concluindo, foi identificado pela primeira vez potenciais variantes genéticas que protegem (*RSPO4*) ou predispõem (*BMP4*) à DISH/CC. A relevância do gene *ABCC6* neste fenótipo necessita de ser provada. É pouco provável que um único gene esteja envolvido no aparecimento de DISH/CC, e por isso a doença parece ser poligénica.

Palavras-chave: Condrocalcinose, DISH, WES, *BMP4*, *ABCC6*, *RSPO4*.

RESUMO ALARGADO

A hiperostose idiopática difusa do esqueleto (DISH, MIM 106400) é uma doença musculoesquelética comum caracterizada pela calcificação progressiva e ossificação de tecidos moles, em particular ligamentos e enteses [1, 2]. Em alguns casos a deposição óssea pode levar a alterações biomecânicas do sistema músculo-esquelético e/ou a formação de massas cervicais obstrutivas [3, 4]. Desconhece-se a prevalência e a incidência exata da DISH, porém sabe-se que é mais frequente no sexo masculino e que a sua prevalência aumenta com a idade, afetando principalmente os indivíduos com mais de 40 anos [5]. Várias evidências sugerem que fatores genéticos estão envolvidos na etiologia da DISH, como a existência de casos familiares com início precoce (na terceira ou quarta década de vida) [29], e a maior frequência da DISH em uma raça específica de cães, a raça boxer [30, 31]. Variantes de suscetibilidade para a DISH foram encontradas nos genes *COL6A1* e *FGF2*, no entanto estas estão localizadas em regiões não codificantes da proteína e são consideradas variantes comuns na população em geral, o que sugere um efeito menor ou não patogénico. Embora os genes *COL6A1* e *FGF2* pareçam estar envolvidos na suscetibilidade da DISH, a genética da DISH é ainda desconhecida.

A DISH pode coexistir com um grande número de outras doenças reumáticas, sendo exemplos destas a ossificação do ligamento lateral posterior (OPLL, MIM 602475) [6], Ossificação do ligamento flavum [8], Espondilite anquilosante (AS) (MIM 106300) [7-21] e Condrocalcinose (CC) [22, 23]. A coexistência da DISH com CC é muito comum na ilha Terceira (Açores) e parece ser uma manifestação endémica. Estudos anteriores, conduzidos pelo nosso grupo, levaram à identificação e caracterização de doze famílias com início precoce de DISH e/ou CC levando o grupo a sugerir que ambas as doenças, designadas como fenótipo DISH/CC, poderiam partilhar o mesmo mecanismo patogénico. Estas famílias parecem apresentar um tipo familiar precoce, autossómica dominante, com um fenótipo que inclui calcificações entesopáticas periféricas e axiais [24]. Um fenótipo similar foi relatado em outros estudos no passado [25, 26]. Estes doentes têm a sua qualidade de vida gravemente comprometida devido ao início precoce e ao fenótipo exuberante.

Este estudo foi realizado nos Açores, um arquipelago português localizado no meio do Oceano Atlântico, numa pequena ilha com apenas 56.467 habitantes, Ilha Terceira (Census, 2011). Em populações isoladas ou com mobilidade reduzida, como é o caso da

Ilha Terceira, existe uma elevada taxa de casamentos consanguíneos e uma alta probabilidade de ancestralidade comum que é particularmente valiosa no mapeamento de genes envolvidos em doenças monogénicas mendelianas e, portanto, investigar o fenótipo DISH/CC nessa população aumenta a probabilidade de identificar o gene causador desta patologia. Na ilha Terceira Açores foi estabelecido um biobanco com produtos biológicos e dados associados para a população da Ilha Terceira. Os Biobancos são essenciais na investigação, por conterem coleções de amostras e dados armazenados de forma organizada. Actualmente, o biobanco dos Açores (AZORBIO) do Serviço Especializado de Epidemiologia e Biologia Molecular (SEEBMO) tem uma colecção de material biológico e dados associados de doentes açorianos de diferentes patologias [27].

O objetivo principal desta tese foi investigar doentes e famílias afetadas com o fenótipo DISH/CC para determinar a sua base genética. Para atingir esse objetivo, foram utilizados diferentes estudos que se encontram divididos ao longo dos capítulos desta tese.

No capítulo 2 foi realizada uma revisão da literatura sobre a genética da ossificação dos ligamentos da coluna vertebral (OSL). Com base na sua análise verifica-se que a contribuição genética para o desenvolvimento destas ossificações parece ser inegável. A existência de casos familiares com início precoce (terceira/quarta década de vida) [1], a existência de modelos animais [2, 3], e a existência de uma grande variedade de genes com associação positiva, essencialmente na OPLL, são evidências que reforçam ainda mais esta predisposição genética. É provável que esta contribuição genética não seja causada por um único gene com grande efeito (padrão de herança mendeliana), mas sim por uma variedade de variantes em diversos genes. Para além disso existem também múltiplos fatores exógenos que podem estar envolvidos na patogénese da doença, mas provavelmente em indivíduos suscetíveis. Portanto, a OSL parece ser genética e multifatorial. A presença de OSL tem também sido associada com inúmeros distúrbios metabólicos de diferentes etiologias. A coexistência de ossificação dos ligamentos da coluna espinhal com alguns transtornos monogénicos tem sido relatada na literatura e estes normalmente estão associados a uma perturbação na homeostasia do cálcio e fosfato, levando-nos acreditar que os genes envolvidos nestes processos são bons candidatos para a etiologia da OSL.

No **capítulo 3** são apresentados os materiais e métodos utilizados ao longo de todo o trabalho. No **capítulo 4**, encontra-se descrito um caso de CC associado à síndrome de Gitelman. A CC é caracterizada pela deposição de sais de cálcio na cartilagem articular, membranas sinoviais e, em alguns casos, nos tecidos moles periarticulares [28]. Os sais

depositados são normalmente compostos por pirofosfato de cálcio desidratado (CPP), embora outros sais de cálcio, como a hidroxiapatite, também possam ser encontrados [28]. Sabe-se que mutações no gene *ANKH*, em algumas famílias, são a causa monogénica de CC articular familiar (CCAL2, MIM 118600). Esse gene está relacionado com o metabolismo do pirofosfato inorgânico (PPi) sendo responsável pelo transporte deste pela membrana. A CC pode ocorrer sob 3 formas: 1) hereditária, 2) esporádica, e 3) associada a doenças metabólicas como hiperparatiroidismo, hemacromatose, doença de Wilson, Síndrome de Gitelman entre outras. A síndrome de Gitelman é uma doença genética renal autossómica recessiva, causada por uma mutação no gene *SLC12A3*, o qual codifica o transportador NCCT (thiazide-sensitive sodium-chloride cotransporter) expresso no túbulo contornado distal do rim. A síndrome é caracterizada por alcalose metabólica, hipocalemia, hipocalciúria e hipomagnesiémia. A hipomagnesiémia está associada a uma redução na concentração de magnésio celular e pertence à lista de causas de CC secundária associada a síndromes metabólicos. A hipomagnesiémia parece favorecer a CC através da elevação intra-articular dos níveis de PPi extracelular e/ou através da redução da solubilidade dos cristais de CPP [4]. Neste estudo foi identificado um caso de CC associada a hipomagnesiémia causada por uma mutação patogénica homozigótica no gene *SLC12A3*. Alguns familiares deste doente são portadores da mutação heterozigótica e apresentavam CC.

No **capítulo 5** foi realizado um estudo de sequenciação de dois genes candidatos ao fenótipo DISH/CC, o gene *LEMD3* e o gene *RSPO4* para verificar uma possível associação com o fenótipo em estudo. Foram identificadas várias variantes no gene *RSPO4*, no entanto não foram observadas diferenças estatisticamente significativas na ocorrência destas variantes genéticas no fenótipo DISH/CC relativamente ao grupo controlo. Duas variantes na região reguladora do gene *RSPO4* (rs146447064 e rs14915407) são significativamente mais frequentes em controlos do que em doentes DISH/CC, indicando que estas podem eventualmente afetar a expressão do gene, conferindo proteção à doença. A variante estudada no gene *LEMD3* é extremamente rara e parece não estar envolvida com o fenótipo em estudo.

No **capítulo 6** desta tese descreve-se o estudo de sequenciação exómica realizado em quatro indivíduos das 12 famílias anteriormente referidas com o objetivo de determinar o possivel gene envolvido no fenótipo DISH/CC, aparentemente autossómico dominante. A sequenciação completa do exoma é uma técnica capaz de identificar rapidamente todas as variantes codificantes no genoma de um individuo. Esta técnica tem sido uma ferramenta

essencial para a deteção de variantes patogénicas causadoras de doenças. Das filtragens efetuadas surgiram vinte e uma variantes genéticas relevantes, em dezassete genes que estão direta ou indiretamente relacionados com a mineralização e/ou ossificação. Identificamos uma associação significativa entre a doença DISH/CC e a variante genética rs17563 no gene *BMP4*, um gene diretamente envolvido na formação óssea endocondral (p = 0,009, OR = 2,331).

No **capítulo 7** descreve-se o estudo do gene *ABCC6*, um gene que surgiu das filtragens da sequenciação exómica. Este gene contém 31 exões e está localizado no cromossoma 16, assim como os seus dois pequenos pseudogenes *ABCC6P1* e *ABCC6P2*. O gene codifica o transportador MRP6 que pertence à família de transportadores da membrana dependentes de ATP. Pensa-se que está envolvido no transporte de adenosinas trifosfato. O MRP6 é composto por 1503 aminoácidos, três segmentos membranares, consistindo em 17 hélices hidrofóbicas e dois domínios de ligação a nucleótidos. A proteína apresenta expressão ubiquitária no entanto é principalmente no fígado e rins que esta se expressa mais. Alterações neste gene e também no gene *ENPP1*, já anteriormente designado como associado à OPLL, estão associadas ao Pseudoxantoma Elasticum (PXE, MIM 264800) e à calcificação arterial generalizada da infância (GACI2, MIM 614473). A PXE é caracterizada por calcificações das fibras elásticas na pele, artérias, trato gastrointestinal e retina. A PXE é geralmente autossómica recessiva podendo ser esporádica ou dominante com penetrância variável. A GACI, doença autossómica recessiva, é caracterizada por calcificações na lâmina elástica interna das artérias musculares e estenose. O gene *ABCC6* foi considerado um candidato ideal porque: 1) mutações genéticas neste gene são a maior causa da PXE, e em alguns casos podem causar GACI, doenças que tal como a DISH/CC causam calcificações ectópicas; 2) a maioria dos casos de GACI são devidos a mutações no gene *ENPP1*, levando-nos a pensar que os dois genes podem ter processos fisiológicos comuns e, para além disso, o gene *ENPP1* foi anteriormente estudado por nós e por outros grupos por poder estar associado à CC; 3) recentemente foi estudado a associação de um transportador de adenosinas - ENT1- com a DISH; como o gene *ABCC6* pode também ser um transportador de adenosinas, leva-nos também a pensar que pode haver algum mecanismo fisiológico comum entre os dois transportadores. A variante rara não sinónima rs41278174 no gene *ABCC6* foi encontrada num doente. A variante é altamente conservada entre mamíferos, e de acordo com os algoritmos utilizados (SIFT e PolyPhen) pode afetar a proteína. Verificámos que esta variante é frequente na ilha Terceira- Açores nomeadamente em controlos masculinos quando

comparados com doentes do sexo masculino (DISH/CC e AS). Esta variante está localizada no domínio transmembranar da proteína MRP6, um domínio essencial na especificidade do substrato de transportadores ABC [25]. Mutações no domínio transmembranar também podem afetar a integração da proteína na membrana celular levando a uma perda de função [26]. A variante rs41278174 poderá alterar a especificidade do transportador MRP6 nos homens, conferindo um efeito protetor através de um mecanismo desconhecido. Foram encontradas outras variantes neste gene mas de acordo com o estudo de associação não apresentam associação positiva com a DISH/CC. Os estudos de expressão por qPCR mostraram que os transcritos de *ABCC6* são pouco expressos em tecidos de cartilagem humana, no entanto verificou-se que nos doentes DISH e CC o gene apresentava expressão superior em relação a um doente controlo. Este aumento de expressão poderá estar envolvido com a formação de cristais de CPP em doentes DISH e CC.

No **capítulo 8** foi realizado uma abordagem comparativa utilizando teleósteos, que revelou que o gene *abcc6* está expresso na pele mas não está associado com a calcificação ectópica das escamas. Além disso, na genómica comparativa o gene *abcc6* só foi encontrado no genoma de vertebrados ósseos, indicando que este gene poderá estar envolvido com inovações específicas dos vertebrados.

Os resultados deste trabalho sugerem que vários genes parecem estar envolvidos na etiologia do fenótipo DISH/CC. A associação positiva encontrada para o gene *BMP4* deverá ser reproduzida com um maior número de doentes para verificar a sua veracidade. A expressão aumentada do gene *ABCC6* encontrada nos doentes DISH e CC deverá também ser confirmada com mais amostras de cartilagem a fim de esclarecer e confirmar se este gene está mesmo envolvido na formação de deposição de CPP nos doentes com DISH e CC.

Palavras-chave: Condrocalcinose, DISH, WES, *BMP4*, *ABCC6, RSPO4,* calcificações ectópicas, variantes genéticas, genes, Açores, PPi, CPP.

LIST OF ABBREVIATURES

A

A- Affected
A1- allele1
A2- allele 2
AA- Amino acids
A-Allelic test
ABC- ATP binding cassete
ABCC6,1,3 - ATP-binding cassete subfamily C, member 6, 1,3
ABCC6P1- ATP-binding cassette subfamily C, member 6 pseudogene 1
ABCC6P2- ATP-binding cassette subfamily C, member 6 pseudogene 2
ABCG2- ATP Binding Cassette Subfamily G Member 2
ABI-SOLiD- Applied Biosystems Sequencing by Oligonucleotide Ligation and Detection
Aca- *Anolis carolinensis*
ACE- Angiotensin I Converting Enzyme
ACVR1- Activin A receptor, type I
AD- Autosomal dominant
Aga- *Anopheles gambiae*
AHSG: Alpha 2-Heremans-Schmid glycoprotein
AIP- Aryl hydrocarbon receptor-interacting protein
Ala- alanine
ALPL- Alkaline Phosphatase, Liver/Bone/Kidney
AMDH- Acromesomelic dysplasia, Hunter-Thompson
Ame- *Astyanax mexicanus*
Amel- *Apis mellifera*
AMER3- APC Membrane Recruitment Protein 3
AMP- Adenosine monophosphate
ANKH- progressive ankyloses protein homolog
ANO6- Anoctamin 6
ANTXR2- Anthrax Toxin-receptor 2
AOMS1,2- Abdominal obesity-metabolic syndrome 1,2
AP2S1- Adaptor Related Protein Complex 2 Sigma 1 Subunit
AR- Autosomal recessive
ARL6IP1- ADP ribosylation factor like protein 6 interacting protein 1
AS- Ankylosing spondylitis
as- altered splicing
ASARM- Acidic serine- and aspartate- rich MEPE- associated motif
ASPN- Asporin
At- Annealing temperature
ATP- Adenosine triphosphate
AVH- Ankylosing Vertebral Hyperostosis
AZORBIO- AZOresBIObank

B

BC- Breast cancer
Bfl- *Branchiostoma floridae*
BID- BH3 Interacting Domain Death Agonist
BMP- Bone Morphogenetic Proteins
BMP2- Bone morphogenetic protein 2
BMP4- Bone morphogenetic protein 4
BMP9- Bone morphogenetic protein 9
BMPR1B- Bone morphogenetic protein receptor type 1B
BOS- Buschke ollendorff syndrome
BSA- Bovine Serum Albumin

Bta- *Bos taurus*

C

C2H2- Cys2His2-like

CASR- calcium-sensing receptor

CC- Chondrocalcinosis

CCAL1- Chondrocalcinosis 1

CCAL2- Chondrocalcinosis 2

CCDC91- Coiled-coil domain containing 91

CCMAR- Centro de Ciências do Mar

CD4 cells- cluster of differentiation 4 cells

CDKN1β- Cyclin-dependent kinase inhibitor 1β

cDNA- Complementary DNA

Cel- *Caenorhabditis elegans*

CFTR- Cystic Fibrosis transmembrane Conductance Regulator

CHISQ- Chi- squared

CHISQ- Chi-squared

Chr- chromosome

CLCN5- Chloride voltage-gated channel 5

CLCNKB- Chloride channel clc-kb

Cluf- *Canis lupus familiaris*

CMDD- Craniometaphyseal dysplasia

cMGP- carboxylated Matrix Gla Protein

Cmi- *Callorhinchus milii*

COL11A2- Collagen Type XI Alpha 2 Chain

COL17A1- Collagen Type XVII Alpha 1 Chain

COL1A1- Collagen Type I Alpha 1 Chain

COL2A1- Collagen Type II Alpha 1 Chain

COL6A1- Collagen Type VI Alpha 1 Chain

COL6A4P1- collagen Type VI, alpha-4, pseudogene 1

COQ7- Coenzyme Q7

CPP- calcium pyrophosphate

CPPD- Calcium pyrophosphate deposition disease

Csa- *Ciona intestinalis*

C-Spine- Cervical spine

Ct- cycle threshold

Ct- Cycle threshold

CTD- Citoplasmatic domain

Cys- cystein

D

Dgg- dragon fish

DISH- Diffuse idiopathic skeletal hyperostosis

DKK1- Dickkopf WNT Signaling Pathway Inhibitor 1

Dla- *Dicentrarchus labrax*

DM- Diabetes mellitus

Dme- *Drosophila melanogaster*

DMP- Deborah Mary Power

DMP1- Dentin matrix acidic phosphoprotein 1

DMSO- Dimethyl sulfoxide

DNA- Deoxyribonucleic acid

DNase- Deoxyribonuclease

DNase- Deoxyribonuclease

Dno- *Dasypus novemcinctus*

Dpu- *Daphnia pulex*

DR- Diabetic retinopathy

DRD2- Dopamine receptor

Dre- *Danio rerio*

DYRK1B- Dual specificity Tyrosine Phosphorylation regulated kinase 1β

E

E- Exon

EDIL3- EGF Like Repeats And Discoidin Domains 3

EDTA- Ethylenediamine tetraacetic acid

ENPP1 /*NPPS*/*PC1*- ectonucleotide pyrophosphatase/phosphodiesterase 1

ENT1- Equilibrative Nucleoside Transporter 1

ER- Endoplasmic reticulum

ERAP1- endoplasmic reticulum aminopeptidase 1

ESR1- Estrogen Receptor 1

EST- Expressed sequence tag

EtOH- Ethanol

ExoSAP-IT- Exonuclease I and Shrimp Alkaline Phosphatase

Ext- extracellular

F

F- Female

FAM- Fluorescein amidite

FGF2- Fibroblast growth factor 2

FGF23- Fibroblast growth factor 23

FGFR1- Fibroblast Growth Factor Receptor 1

FHH- Familial Hypocalciuric hypercalcemia

FLNC- Filamin C

FOPNL- FOP related protein

FRZB- Frizzled-related protein 1

FZD5- Frizzled Class Receptor 5

G

Gac- *Gasterosteus aculeatus*

GACI- Generalized arterial calcification of infancy

GCM2- Glial cells missing Homolog 2

GDF5- Growth/differentiation factor 5

GERP- Genomic Evolutionary Rate Profiling

Gga- *Gallus gallus*

GH- Growth hormone

Gln- Glutamine

GLUT9- Glucose transporter 9

Gmo- *Gadus morhua*

GNA11- G Protein Subunit Alpha 11

GNAS1- Guanine nucleotide binding protein, alpha stimulating

GPR10- G protein-coupled receptor 101

GS- Gitelman syndrome

H

HAPLN1- Hyaluronan And Proteoglycan Link Protein 1

HDL- High density lipoprotein

HGD- Homogentisate 1,2-Dioxygenase

HGMD- Human genetic mutation database

HiDi formamide- highly deionized formamide

His- histidine

HLA- Human Leukocyte antigen

HLA-B- Human Leukocyte antigen B

HLA-DQA2- major histocompatibility complex, class II, DQ alpha 1

HOA1- Hydroxyacid oxidase 1

Hro- *Helobdella robusta*

Hsa- *Homo sapiens*

HSEIT- Hospital de Santo Espirito da Ilha Terceira

HWE- Hardy –Weinberg equilibrium

I

i- intron

IBD- Identity by descent

IBSP- Integrin binding sialoprotein

IFNG-Interferon, Gamma

IGF1- Insulin-like growth factor 1

IL-1- Interleukin-1

IL-15RA- Interleukin 15 Receptor Alpha

IL-17- Interleukin-17

IL-1β- Interleukin 1 Beta

IL-23- Interleukin-23

IL-23R- Interleukin-23 receptor

IL-6- Interleukin-6

IL-8- Interleukin-8

IPD-IMGT/HLA- Immuno Polymorphism Database- ImMunoGenetics information system for Human Leucocyte Antigene

J

JAG1- Jagged 1

JBA- Jácome Bruges-Armas

K

K- Potassium

Kb- Kilobases

L

LaTaq- Long and accurate taq

LBP- Low back pain

Lch- *Latimeria chalumnae*

LEMD3- LEM domain containing 3

LEP- Leptin

LEPR- Leptin receptor

Lji- *Lottia gigantea*

Loc- *Lepisosteus oculatus*

LRP6- LDL Receptor Related Protein 6

LRP6- Low density lipoprotein receptor related protein 6

L-Spine- Lumbar spine

M

M- Males

MAF- Minor allele frequency

MATN3- Matrilin-3

MCP- Metacarpophalangeal

Mdo- *Monodelphis domestica*

MEN1- Menin 1

MGP- Matrix Gla Protein

MHC- Major Histocampatibility Complex

MIF- Macrophage inhibitory factor

MIM- Mendelian Inheritance in Man

min- minutes

miRNA- micro ribonucleic acid

miRNA SNP- micro ribonucleic acid related single nucleotide database

mRNA- messenger ribonucleic acid

Mmu- *Mus musculus*

MODY- Maturity-Onset Diabetes of the young

MRNA- Messenger ribonucleic acid

MRP1- Multidrug resistance protein 1

MRP3- Multidrug resistance protein 3

MRP6- Multidrug resistance protein 6

MTP- Microsomal triglyceride transfer protein

MYH11- Myosin heavy chain 11

N

NA- Not applicable

NCBI-National Center for Biotechnology Information

NCCT- sodium chloride cotransporter

NCX1- Sodium/calcium exchanger

NDE1- Neurodevelopment protein 1

NDNC4- Nonsyndromic congenital nail disorder-4

NFB1- Nucleotide Binding Fold 1

NFB2- Nucleotide Binding Fold 2

ng- nanograms

NGS- Next generation sequencing

Nm: nanometers

NOMO3- Nodal modulator 3

NPT3- Sodium phosphate transporter protein 3

NSAIDS- Nonsteroidal anti-inflammatory drugs

NSCL/P- Nonsyndromic cleft lip with or without palate

NTC- No template control

O

LF- Ligament flavum

OA- Osteoarthritis

Oan- *Ornithorhynchus anatinus*

Ola- *Oryzias latipes*

OLF- Ossification of the ligament flavum

OMIM- Online Mendelian Inheritance in man

Oni- *Oreochromis niloticus*

OPG- Osteoprotegerin

OPLL- Ossification of the Posterior longitudinal ligament

OR – Odds ratio

ORF- Open reading frame

OSL- Ossification of Spinal Ligaments

P

P- Proband

PCR- Polymerase chain reaction

PCSK1- Proprotein convertase subtilisin/kexin 1

PD- Paget disease

PDB4- Paget disease of bone 4

PHEX- Phosphate regulating endopeptidase homolog, X-linked

Pi- inorganic phosphate

PLCG2- Phospholipase C Gamma 2

Pm- p value maternal

Pma- *Petromyzon marinus*

POH: Progressive osseous heteroplasia

PolyPhen- Polymorphism Phenotyping v2

POMC: Pro-opiomelanocortin

Pp- pvalue paternal

PPi- inorganic pyrophosphate

PPP2R2D- Protein Phosphatase 2 Regulatory Subunit B delta

PRKAR1A- Protein Kinase CAMP-Dependent Type I Regulatory Subunit Alpha

Pro- Proline

PTCH1- Patched 1

PTH- Parathyroid hormone

PTHR2- Parathyroid Hormone 2 Receptor

Ptr- *Pan troglodytes*

PXE- Pseudoxanthoma elasticum

Q

qPCR- Quantitative Polymerase chain reaction

R

r.p.m.- rotations per minute

RA- Rheumatoid arthritis

RANKL- receptor activator of nuclear factor-kappaB ligand

RDD- RNase-free DNase set

Ref- References

RIN- RNA Integrity Number

RLT- RNeasy lysis buffer

RNA- Ribonucleic acid

RNA- Ribonucleic acid

RPS18- GRibosomal Protein S18

RR- Regulatory regions

RRM- RNA recognition motif

RSPH9- radial spoke head 9 homolog

RSPO2- R-spontin 2

RSPO4- R-spontin 4

RSPS15A- Ribosomal protein S15A

RT-PCR- Reverse transcription polymerase chain reaction

RUNX2- Runt-related transcription factor 2

RXRB- Retinoid X Receptor Beta

S

SEEBMO- Specialized Service of Epidemiology and Molecular Biology

SETS- Sequencing by oligonucleotide ligation and detection Experimental Tracking Systems

SIFT- Sorting Intolerant From Tolerant

SLC12A3- Solute carrier family 12 member 3

SLC17A3- Solute carrier family 17 member 3

SLC29A1- Solute carrier family 29 member 1

SLC2A9- Solute carrier family 2 member 9

SLC34A3- Solute carrier family 34 member 3

SNP- Single nucleotide polymorphism

SNV- Single Nucleotide Variant

SPDA1,2,3- Spondyloarthropathy, Susceptibility To, 1,2,3

SPL- Splicing

Spu- *Strongylocentrotus purpuratus*

SQSTM1- Sequestosome-1

STK38L: serine/threonine kinase 38 like

T

T- Trend test

T1D- Type 1 Diabetes

T2D- Type 2 Diabetes

Tca- *Tribolium castaneum*

TDT- transmission disequilibrium test

TGFBR2- Transforming Growth Factor, Beta Receptor II

TGF*β*- Transforming Growth Factor, Beta

TGFβ1- Transforming Growth factor Beta 1

TGFβ3- Transforming Growth factor Beta 3

Th17- T helper 17

THR- Tip hip replacement

THR- Total hip replacement

TMD- transmenbrane domain

TNAP- Tissue-nonspecific alkaline phosphatase

TNF- tumor necrosis factor

TNFRSF11A- TNF Receptor Superfamily Member 11a

TNFRSF11B- TNF Receptor Superfamily Member 11b

Tni- *Tetraodon nigroviridis*

T-Spine- Thoracic spine

Twy- Tiptoe Walking Yoshimura

U

ucMGP- un-carboxylated matrix Gla Protein

UHM- U2Af homology motif kinase 1

UN- Unaffected

UTR- Untranslated region

V

VDR- Vitamin D Receptor

VKORC1- Vitamin K epoxide reductase complex subunit 1

vs- versus

W

WES- Whole exome sequencing

WNT- Wingless

Wt- Wild type

X

XLD- X-linked dominant

XLR- X-linked recessive

Xma- *Xiphophorus maculatus*

Xtr- *Xenopus tropicalis*

XYLT1- Xylosyltransferase 1

Y

YWHAZ- Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

Z

ZFR- Zucker fatty rat

ZNF687- Zinc Finger Protein 68

LIST OF CONTENTS

TABLES

SUPPLEMENTARY TABLES

FIGURES

SUPPLEMENTARY FIGURES

CHAPTER I: INTRODUCTION

1. INTRODUCTION

1.1 Background of the study

The starting point of this thesis was the identification of many families, from Terceira Island, Azores, that may represent an early onset, autosomal dominant, familial type of pyrophosphate arthropathy, with a phenotype that includes peripheral and axial enthesopathic calcifications. A detailed radiological analysis of these families showed a high level of ectopic calcification especially in elbows (82.9%) and spine (81.4%). The concurrence of spinal ossifications, resembling Diffuse Idiopathic Skeletal Hyperostosis (DISH), and CPPD Chondrocalcinosis (CC), in many of those patients, suggested a shared pathogenic mechanism [24]. For a number of years the possible major gene involved in the aethiopathogenesis, of the then designated DISH/CC phenotype, has been investigated using different strategies, including whole exome linkage and an Identity-by-state study, and two zones, in chromosomes 12 and 20, seemed relevant for further investigation [29]. For this purpose a biobank with biological products and associated data was established for the population from the Terceira Island in the Azores. Biobanks are essential in research, by having collections of samples and data stored in an organized manner. At the moment the biobank Azores (AZORBIO) of the Specialized Service of Epidemiology and Molecular Biology (SEEBMO) has a collection of biological material and associated data of Azorean patients with different pathologies [27]. The Azores archipelago (Portugal) is located in the middle of the Atlantic Ocean, 1500 km from the European mainland and is formed by nine islands of volcanic origin. The islands are grouped according to their geographic positions: Eastern (São Miguel and Santa Maria), Central (Terceira, Faial, Pico, Graciosa and São Jorge) and Western (Flores and Corvo) [24, 30] (Figure 1-1). The Azores were officially populated in 1439 and have approximately 246,746 inhabitants distributed in a very asymmetric way among islands. Terceira is a relatively small island with only 56.467 inhabitants (Census, 2011). Isolated populations or populations with reduced mobility, as is the case of Terceira Island, have proven particularly valuable for the purposes of mapping genes involved in Mendelian monogenic disorders and thus, investigating this phenotype in this population it was reasoned would increase the likelihood of identifying the causative gene mutation.

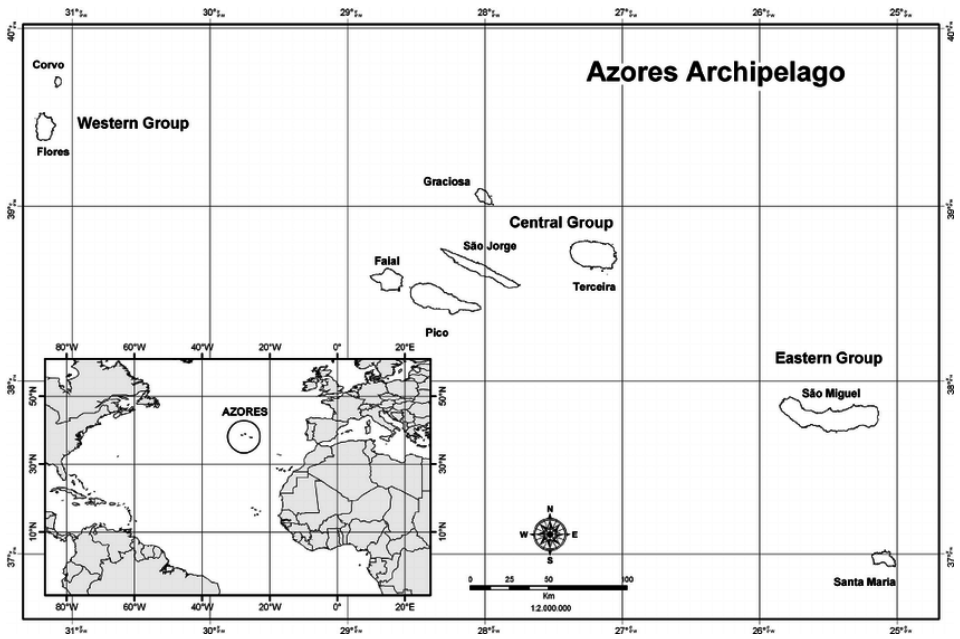


Figure 1-1. Geographic location of the Azores. The islands are grouped according to their geographic positions in Eastern, Central and Western. Taken from Santos et al, 2009 [30].

1.2 Objectives of the thesis

The main objective of this thesis was to proceed with the investigation of the genetic basis of the DISH/CC phenotype making use of Next Generation Sequencing technology, together with association and expression studies.

The studies presented in this thesis were guided by the following objectives:

- Investigate the association of CC with the metabolic disorder- Gitelman Syndrome;
- Investigate the candidate genes *RSPO4* and *LEMD3* genes, derived from a previous analysis of identity-by-descent sharing across four families with CC;
- Select the best candidate genes in WES results from 4 unrelated DISH/CC patients;
- Perform case/control and expression studies on the best candidate genes;
- Characterize the candidate gene in terms of origin and evolution.

On completion of this thesis I expect to contribute to the identification of genetic factors involved in the DISH/CC phenotype.

1.3 Thesis outline

The **Chapter 2** introduces the basic knowledge of what is known about the genetics of ossification of spinal ligaments- DISH, OPLL and OLF, and focusses on the main disorder investigated in this thesis: DISH. **Chapter 3** provides a detailed presentation of the material and methods used in the thesis. The association of CC with Gitelman Syndrome through the genetic analysis of the *SLC12A3* gene is presented in **chapter 4**. **Chapter 5** covers the possible role of *RSPO4* and *LEMD3* genetic variants in the aetiology of DISH/CC. The whole exome sequencing study performed in order to detect genetic variants that are expected to have a potentially damaging effect on proteins with functions related to calcification and/or ossification is reported in **chapter 6**. In **chapter 7** the candidate gene *ABCC6* is investigated using case/control and expression studies to verify the association with the phenotype under study. Finally, the characterization and comparison of the *ABCC6* gene in metazoans including humans using "in silico" methodologies is presented in **chapter 8**. The **chapter 9** presents the general discussion and conclusions of this thesis. The final chapter contains the bibliography, followed by appendix with an article publication.

CHAPTER II: LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 Introduction

The spine is a columnar structure in the center of the body composed by spinal bones (vertebrae) and inter-vertebral discs. It is supported by spinal ligaments (flexible band-like structures), which include the anterior and posterior longitudinal ligaments, ligament nuchae and the yellow ligament (ligamentum flavum) of the spine [31]. There is a group of diseases characterized by the ossification of spinal ligaments (OSL); the anterolateral spinal ligament [Diffuse Idiopathic Skeletal Hyperostosis (DISH; MIM 106400)], the posterior longitudinal ligament [Ossification of the Posterior Longitudinal Ligament (OPLL; MIM 602475)] and the ligament flavum [Ossification of Ligamentum Flavum (OLF)]. In some cases OPLL, DISH and OLF co-occur in the same patient [32] suggesting possible common aetiopathogenic factors. Genetic links in OPLL, DISH and OLF have been investigated and several papers cite or allude to genetic factors as playing a role in the aetiology of this diseases [33-35]. There are reports in the literature that describe familial cases of DISH and OPLL which further strengthen this genetic association. Although the evidence of a genetic predisposition in all the three diseases have been described, very few studies in DISH and OLF appear in the literature, only OPLL has been extensively investigated. However, the studies that have looked at the possible genetic links are still inconclusive and the aetiology of these diseases remains unknown. The main objective of this study is to investigate genetic variants associated with DISH susceptibility. Therefore, in this thesis, the genetic mechanisms already involved in OPLL and OLF aetiology will be explored in detail since they can give insights into the pathogenesis of DISH.

2.2 Diagnosis

DISH (DISH; MIM 106400) is the current terminology for a systemic non inflammatory disorder reported in 1925 by Knaggs [36] and later described by Forestier and Routes-Querol in 1950 [37]. This disorder has had a variety of denominations in the literature throughout the years, due to the diverse phenotypes encountered. It is a common condition amongst the elderly characterized by calcification and ossification of the anterior longitudinal ligament affecting, in particular, the right side of the spine with preservation of the intervertebral disc space. Whilst spinal involvement in DISH is nearly

universal, extraspinal sites, such as the elbow, shoulder, hip, knee and heel are very common [1, 5, 22, 23, 38]. The diagnosis of DISH is established using radiographies. There are two main diagnostic criteria sets to identify definite, probable or possible DISH (Table 2-1). Resnick [39] defined the first set of criteria that were, some years later, revised by Utsinger [5] for epidemiological purposes. Criteria are indicated in the following table:

Table 2-1. DISH diagnostic criteria.

			Resnick Criteria [39]	**Utsinger Criteria [5]**
Definite	**1**		Presence of flowing calcification and ossification along the anterolateral aspect of at least four contiguous vertebral bodies with or without associated localized pointed excrescences at the intervening vertebral body-intervertebral disc junctions.	Continuous ossification along the anterolateral aspect of at least four contiguous vertebral bodies, primarily in the thoracolumbar spine. Ossification begins as a fine ribbon-like wave of bone but commonly develops into a broad, bumpy, buttress-like band of bone.
Probable	**Possible**	**2**	The presence of relative preservation of intervertebral disc height in the involved vertebral segment and the absence of extensive radiographic changes of "degenerative" disc disease, including vacuum phenomena and vertebral body marginal sclerosis.	Continuous ossification along the anterolateral aspect of at least two contiguous vertebral bodies.
	Possible	**3**	The absence of apophyseal joint bony ankylosis and sacroiliac joint erosion, sclerosis or intraarticular osseous fusion.	Symmetrical and peripheral enthesopathy involving the posterior heel, superior patella or olecranon, with the entheseal new bone having a well-defined cortical margin.

According to the criteria, the probability of DISH is as follows: 'definite' if criterion 1 is present, 'probable' if criteria 2 and 3 are present and 'possible' if criterion 2 or 3 is present; particularly if calcaneal spurs occur together with olecranon or patellar spurs. Exclusion criteria include: abnormal disc space height in the involved areas and/or apophyseal joint ankylosis. Resnick criteria number 3 has an exclusion factor based on the erosions, sclerosis or fusion of sacroiliac joints. This helps to exclude patients with Ankylosing Spondylitis (AS), a disease that can be confused with DISH. The third factor for exclusion of DISH diagnosis was withdrawn by Utsinger because differentiation of these two disorders should be possible with lateral and antero posterior axial x-rays. Recently efforts have been made to revise the definition of DISH in order to incorporate the current knowledge about DISH, however a new definition of DISH is still under debate [40]. Despite the need for a new definition of DISH, the criteria proposed by Utsinger are still universally accepted and widely used in the literature and will be used in this thesis.

OPLL (OPLL; MIM 602475) is characterized by ectopic hyperostosis and calcification of the posterior longitudinal ligament at the cervical, thoracic and lumbar spine [41]. In OPLL patients, the cervical spine (70%) is the most commonly affected, followed by the thoracic (15%) and lumbar (15%) spine [42, 43]. Some patients present myelopathy and/or radiculopathy due to chronic compression of the spinal cord and nerve roots. Symptoms of myelopathy are more severe in thoracic OPLL than in cervical OPLL due to the narrow canal, rigidity of the thoracic spine, tenuous blood supply, and inability of the spinal cord to resist much compression [42]. OPLL is diagnosed on lateral plain radiographs as an abnormal radiopacity along the posterior of the vertebral bodies [44], however because of overlying osseous structures, it is important to obtain magnetic resonance images to successfully diagnose OPLL [45]. OPLL is classified in four ossification types: continuous, segmental, mixed and localized or other [44, 46, 47]. The segmental is the most common and involves the ossification behind each vertebral body; the continuous type is an ossified mass that spans several vertebral bodies; the mixed type is a mixture of both continuous and segmental types and the localized or other type the ossification is localized to the intervertebral disk space without involvement of the vertebral body [44].

OLF, also known as ossification of the yellow ligament, is associated with serious neurologic symptoms including thoracic myelopathy and spinal stenosis [48]. The calcification is confined to the ligamentum flavum (LF) and does not extend to the closed spinal bony arch [49]. CPP and hydroxyapatite have been positively identified and are the main players in the calcification of LF [50, 51]. According to Mwaka and collaborators [52] CPP in the cervical LF seems to progress with reduction in elastic fibers, increase in collagen fibrils in the matrix, and migration of metaplastic hypertrophic chondrocytes. The lower thoracic spine is the most affected region, however several cases of cervical, upper thoracic, and lumbar areas have been reported [53, 54]. Cervical radiography, tomography, and computed tomography are useful for diagnosis, however histological examination of the calcified mass using light microscopy, scanning electron microscopy, and x-ray diffraction analysis are essential for the definitive diagnosis [49].

2.3 Epidemiology

The reported epidemiology of DISH differs in the literature. Cassin et al [55] assessed 1000 African blacks aged older than 40 years and reported that the DISH prevalence was

3.8% in males and 4.2% in females. Another study analysed the data from two large American Midwest metropolitan hospital populations with 1363 individuals and reported a prevalence of DISH of 25% in males and 15% in females over 50 years of age and 35% in males and 26% in females over 70 years of age [56]. Holton et al [57] postulated that the prevalence of DISH was 42% in a group of 298 males aged over 65 from the general population. A recent study of 558 Japanese found that the prevalence of DISH was 17.6% using x-ray and 27,2% using computed tomography [58]. The exact prevalence and incidence of DISH is actually unknown, and a reliable estimate of the prevalence of the disease in the general population is difficult due to the benign nature of the condition. The affected individuals do not seek medical care and normally are diagnosed during the examination of other medical conditions [57]. However, it is well known that DISH is more frequent in males and its prevalence increases with age, affecting mainly subjects over the age of 40 [5]. Furthermore DISH seems to have a higher prevalence in developed countries [59], although this predominance might be due to the more frequent use of advanced radiological examinations in developed countries than in undeveloped countries.

OPLL can be found in any population however it is more common in Asian populations, in particular amongst Japanese with a prevalence of 2 to 4% as compared with 0.01 to 2% in non-Asian populations [60]. Men are 2.5 times more likely to develop OPLL than women [41] and the age of onset may be in the fifth decade of life [61], although in some studies no association between age and the presence of OPLL was found [41]. This lack of consensus can be explained by the fact that the study which didn't find any association probably used a group of patients with asymptomatic OPLL, while the other study only involved patients with symptomatic OPLL, already diagnosed.

OLF affects populations worldwide but there is a higher prevalence in east Asian ethnic groups, especially the Japanese, with the incidence of 12% in thoracic OLF (15% in men and 7.7% in women) [62]. OLF is common in the 6^{th} to 7^{th} decades [46].

2.4 Evidences for a genetic aetiology

Genetic links in OPLL, DISH and OLF have been investigated and several papers cite or allude to genetic factors as playing a role in the aetiology of these diseases [33-35]. The

reports in the literature that describe familial cases of DISH and OPLL and the existence of animal models further strengthen this genetic association.

2.4.1 Familial cases

The genetic predisposition to OPLL and DISH is supported by several reports of familial incidence of DISH, and by studies relating a relative recurrence risk of up to 26.1% in parents of OPLL patients and 28.9% in siblings [63].

Reports of familial DISH are uncommon in the literature. One report, dating from 1969, describes a family of Greek Cypriot ancestry with eight individuals showing signs of Ankylosing Vertebral Hyperostosis (AVH) by the third decade. Only three of them had backache as symptoms. All the individuals affected with AVH also shown tylosis (punctuate hyperkeratosis) and the mean age of individuals affected was 31 years old. Six members of this family were affected only with tylosis. Axial skeleton X-ray examination of the eight affected family members showed ossification of paraspinal ligaments, especially in lower thoracic region. There were also large osteophytes with preservation of disk space and marginal sclerosis of the sacroiliac joint. Laboratory results were all normal, the authors mention normal calcium and carbohydrate metabolism and glucose tolerance tests. Obesity was present in most individuals. Weight, in the opinion of the authors, could not account for all the x-ray changes since there was one affected individual with normal build. There was one other case of an individual with tylosis, normal spine and gross obesity. Beardwell et al suggested that the co-existence of tylosis with AVH could indicate a possible genetic link between these two disorders [64]. This link was never confirmed being this association an occasional finding. Another report of familial DISH was published in 1985 by Abiteboul et al [65], where the authors describe two families, one of French Canadian origin and another of Italian origin. French Canadian family is composed of 3 brothers and one sister definitely affected by AVH developing radiological changes by the 4^{th} decade of age. Two other are probably affected with AVH. None of the individuals had diabetes. Italian family was first identified after a coxofemoral surgery of a 71 years old woman. Her sister was submitted to the same surgery when she was 82 years old. Former patient had five daughters, all of them observed by the authors of this study. Two of them showed radiological manifestations of the AVH. Other 2 sisters had more modest phenotypes being classified as probable AVH. None of these individuals was HLA-B*27, was obese or had diabetes. There is another report relating a family with striking cervical disease without extensive dorsal

involvement and normal sacroiliac joints. Inflammatory markers were all normal and none of the individuals of this family was HLA-B*27 positive. The authors denote the unusual phenotype and refer to the difficulty of classifying this condition as DISH [66].
In 2006, Bruges-Armas et al [23] reported twelve familial cases identified on the island of Terceira (Azores, Portugal), multiply affected with DISH and/or chondrocalcinosis (CC). These families may represent a familial type of pyrophosphate arthropathy with a phenotype that includes peripheral and axial enthesopathic calcifications. These findings, according to the authors' suggestion, support the hypothesis that both disorders may share common aetiopathogenic factors. One hundred and three individuals from the twelve unrelated families were assessed. Radiographs were taken of all the individuals including x-rays of the dorsolumbar, pelvis, knees, elbows and wrists, and all cases were screened for known associations of CC. Ectopic calcifications were identified in seventy patients. Axial, elbow, knee and metacarpophalangeal (MCP) pain and/or swelling, deformity and radiographic enthesopathic changes were the most frequent findings/symptoms. Elbow and MCP periarticular calcifications were observed in 35 and 5 patients respectively, and CC was identified in 12 patients. Fifteen patients had sacroiliac disease on computed tomography - ankylosis or sclerosis. 52 patients could be classified as definite (17%), probable (26%), or possible (31%) DISH. Concomitant DISH and CC was diagnosed in 12 patients. Pyrophosphate crystals were identified from knee effusions in 13 patients. The pattern of disease transmission was compatible with an autosomal dominant monogenic disease and the mean age for to develop the disease was 38 years. A recent paleopathological study mentions the co-existence of gout and DISH in the medici Grand Dukes Cosimo I and Ferdinand I [67]. Studies on these families would be very useful and would surely sum up important information related to DISH aetiopathogenesis.

Reports of familial OPLL are scarce but they also occur in the literature. The prevalence of OPLL is much greater among family members of patients with OPLL than in the general population [63, 68], which indicates a strong familial predisposition to OPLL. Familial aggregation of OPLL was first demonstrated by Terayama et al in 1989 [63] in a study assessing 1030 relatives of probands with cervical OPLL in 347 families. The authors found that OPLL was observed in 26.15% of the parents and 28.89% of the siblings of the probands. In this study, the relative risk of first degree relatives developing OPLL are greater than five times that of the expected incidence in the general population. Another study looking to 100 patients and family members with OPLL found a

prevalence of 27% with a relative risk seven times that of the general population [69]. Because the segregation rate in the siblings is higher and the higher prevalence of OPLL in the parents, both authors suggested that OPLL is possibly controlled by autosomal dominant inheritance. In contrast, Hamanishi et al [68] reported one OPLL family with a suspected autosomal recessive trait, but indicated that it was not possible to exclude that it was a dominant trait.

Altogether, familial studies suggest there is a genetic predisposition, however the mode of inheritance for OPLL is not well defined, since the segregation analysis in families support both autosomal dominant and autosomal recessive patterns of inheritance. According to Koga et al [70] the mode of inheritance is obscured by a lack of large families, the late onset of the disease, a sex difference, and environmental effects. Other OPLL familial cases exist in the literature, although they do not indicate the mode of inheritance. Tanabe et al [71] in 2002 report a case of familial thoracic OPLL in Caucasian siblings and Kim et al [72] used OPLL families to find genetic association between *BMP-2* and *COL6A1* polymorphisms.

As far as we known, there are no reports in the literature that describe familial cases of OLF.

2.4.2 Animal models

Several genes have been associated in the regulation of the biomineralization process. In this section genes involved in the pathological mineralization of the axial skeleton in animal models are presented. In recent years, the use of animal models has permitted specific genes to be manipulated and is a powerful tool for the identification of genetic determinants in ectopic calcification disorders. The improvement of strategies to study genetic mutations affecting the skeleton have made it possible the precisely evaluate the role of many different genes and proteins.

2.4.2.1 Animal models for DISH

1) Natural cases

Some naturally cases of DISH disease have been observed in some animals [73-76]. Spinal hyperostosis similar to DISH has been described in canine cases [77, 78] and are more frequent in boxer breed. As in human cases, the disease is more common in older male animals [73]. Similar cases of DISH were reported by Ghazanfar et al [79] in a

fighting Bulldog and by Kornmayer et al [80] in a Weimaraner breed. However, DISH can also occur in smaller breed dogs, and was identified in a 5 year-old, female Shih-tzu [81]. The high prevalence of a specific disease in a certain dog breed and its absence in other breeds, is suggestive of a genetic mechanism [82], and thus these breeds may serve as an animal model for DISH. A recent study identified DISH in a nine years old cat which shows contiguous smooth new bone formation ventral and lateral to the vertebral extending from the cranial thoracic area to the lumbosacral junction, and appearing similar to canine DISH [75].

2) ENT1$^{-/-}$ mice

Warraich et al [83] reported that mice ENT1$^{-/-}$, lacking ENT1 exhibit progressive ectopic mineralization of the upper thoracic and cervical spinal cord resembling human DISH. Furthermore these mice had a significant reduction in the expression of *Enpp1*, *Ank* and *Alpl* genes in intervertebral discs. Another study [84] using ENT1$^{-/-}$ mice focused on the lower portion of the spine and femur, demonstrated that these mice exhibited reduced bone density in the lower half of the spinal column as well as in the midshaft of the femur. Furthermore these authors confirmed the previous findings of Warraich et al [83] that ENT1$^{-/-}$ mice have osteoid formations in the upper portion of the spinal column (thoracic and cervical spinal cord).

In humans *ENT1* gene, also known as solute carrier family 29 member 1 (*SLC29A1*), is one of the four members of equilibrative nucleoside transporters that maps on chromosome 6 (6p21.1). The gene encodes a transmembrane glycoprotein, which transfers hydrophilic nucleosides, such as adenosine, across the plasma membrane (equilibrative transport) [85]. The protein is ubiquitously expressed and involved in purine metabolism transporting the majority of adenosine transport across the plasma membrane. Recently adenosine signaling has been shown to regulate bone remodeling [86]. A search in Ensembl database (http://www.ensembl.org/index.html) reveals that several genetic variants in this gene exist, although none are associated with a human phenotype or disease, with the exception of a mutation (T647C) described by Kim et al [87] that is involved in the development of alcoholism with increased risk of alcohol withdrawal-induced seizures.

2.4.2.2 Animal models for OPLL

1) tiptoe-walking-Yoshimura (twy) mouse

The spinal hyperostotic twy mouse is a naturally occurring mutant that exhibits OSL similar to human OPLL [88], which served as a model for human OPLL disease [89]. Ossification occurs not only in the spinal ligaments but also in various soft tissues such as joint capsules, tendon enthesis, chondral tissues, and peripheral ligaments [89]. The accelerated bone formation characteristic of *ttw* mice is caused by a nonsense mutation in the *Enpp1* gene that causes deficiency in its expression and protein activity. It was thought that the dysfunction of Npps, which has a predicted truncation of the gene product, resulting in the loss of more than one-third of the native protein [89]. The *Enpp1*$^{-/-}$ knock-out mouse has diminished bone density with calcification of joints and vertebrae [90].

ENPP1 (also known as NPPS or PC-1) is one of the seven members of the ectonucleotide pyrophosphate\phosphodiesterase family that maps to chromosome 6 (6q22-q23) [91]. The gene encodes a membrane bound glycoprotein (NPP1) [92], that regulates bone mineralization by hydrolyzing extracellular nucleotide triphosphates (ATP) to produce pyrophosphate [93]. Since NPP1 generates pyrophosphate it serves at least in part as a physiological inhibitor of calcification. The protein occurs in a large variety of tissues, including bone and cartilage, where it occurs in osteoblasts and chondrocytes respectively [94, 95]. In humans, mutations in the *ENPP1* gene are responsible for Generalized arterial calcification of infancy (GACI; MIM #208000), a severe disease characterized by progressive calcification of the internal elastic lamina of muscular arteries and stenosis due to myointimal proliferation [96]. More recently GACI disease has been related to pseudoxanthoma elasticum (PXE; MIM #264800), since in some GACI cases, mutations in *ENPP1* can also cause typical pseudoxanthomatous skin lesions and angioid streaks of the retina [97]. A mouse model for GACI disease, the spontaneous *asj-2J* mutant mouse with the V246D missense mutation in the *Enpp1* gene, has some similarities with ttw mouse. In the *asj-2J* mutant mouse the mutant Enpp1 protein is absent from the liver, and this causes reduced PPi levels in the plasma, and consequently extensive mineralization of a number of tissues, including blood vessels [98]. Mutations in the *ENPP1* gene also causes hypophosphatemic rickets [99] and Cole disease [100].

2) ZFR mouse

The ZFR (Zucker fatty rat) is a murine model originally used for studies of obesity, hyperinsulinemia, hypercholesterolemia and hyperlipidemia. This mice had ossified spinal ligaments, mainly in the thoracic spine and the ossification is histopathologically similar to human OPLL [101]. A molecular variant in the leptin receptor (*LEPR*) gene is the genetic cause in ZFR rats [102].

LEP gene maps on chromosome 7 (7q32.1) and is closely related to bone metabolism, since leptin is a powerful inhibitor of bone turnover *in vivo* [103]. According to Elefteriou et al, leptin is an adipocyte product both necessary and sufficient to control bone mass, since increasing serum leptin levels, reduced bone mass. Conversely, reducing serum-free leptin levels by overexpressing the soluble receptor for leptin increased bone mass [103]. According to Liu et al [104] leptin promoted differentiation of vascular smooth muscle cells from female mice into osteoblasts by increasing RANKL expression. In humans, mutations in this gene cause severe obesity, and morbid obesity with hypogonadism [105].

2.4.2.3 Other animal models with OSL

The *ank* mice shows generalized arthritis associated with extensive hydroxyapatite deposition in articular cartilage and synovial fluid; they also present spinal, peripheral joint, and ligament bony ankylosis and calcification of arteries [106, 107]. The *ANKH* gene maps on chromosome 5 (5p15.1) and encodes a multipass transmembrane protein ANK (492 aminoacids) that transports intracellular inorganic pyrophosphate (PPi) to the extracellular milieu [106], where it acts as a potent inhibitor of mineralization [108]. The Expression Atlas (https://www.ebi.ac.uk/gxa/home) reveals that this protein is expressed in more than 30 different tissues and cells including the eye, brain, lung, kidney, skin and cartilage.

In humans, mutations in this gene have been associated with autossomal dominant craniometaphyseal dysplasia (CMD; MIM#123000) [109] and chondrocalcinosis (CCAL2; MIM #118600) [110].

Zebrafish have been used to study vertebrate mineralization since they share many of the basic features of chondrogenesis and osteogenesis with higher vertebrates [111-118]. In common with humans and mouse, the *enpp1* mutant zebrafish (dragonfish) develops ectopic calcifications in a variety of soft tissues including skin, eye, cartilaginous

elements, the heart, intracranial space and the notochord sheet [114]. Two other zebrafish mutants that display defective skeletal biomineralization, caused by changes in phosphate/pyrophosphate homeostasis in the embryos include *nob* (no bone, mineralization fails) and *dragonfish* (*dgf*), that has ectopic mineralization of the axial skeleton with apparent fusion of the mineralized vertebral centra and bone nodules at characteristic positions of the cleithrum [119]. The *nob* phenotype is caused by a mutation in the ectonucleotidase *entpd5* gene, which has a crucial role in providing sufficient levels of phosphate through hydrolyzing nucleoside triphosphates and diphosphates [119], *Dgf*, in turn, encodes Enpp1 [120]. According to Huitema et all [119], a stringently controlled balance between Entpd5 and Enpp1 activities determines the level of mineralization through controlling the ratio of inorganic phosphate (Pi) to pyrophosphate in the microenviroment of osteoblasts.

Mutations in the zebrafish orthologue *abcc6a* (ATP binding cassete subfamily C, member 6a) gene results in extensive hypermineralisation of the axial skeleton [113]. The ablation of *abcc6a* gene interfered with normal fish development with pericardial edema and a curled tail and was associated with fish death [111]. Another zebrafish mutant, *gräte* (*grt; abcc6ahu4958*) had a mutation in *abcc6a* gene, which has been associated with the regulation of tissue calcification [113]. The *gräte* mutant has excessive mineralisation in the craniofacial and axial skeleton [113]. In zebrafish the *abcc6a* gene is strongly expressed at the site of mineralisation and secretes ATP from cells increasing PPi locally, in contrast with the hepatically derived PPi in mammals. The authors suggested that zebrafish *Abcc6a* is one of several sources of nucleotides for *ENPP1*. In humans, the *ABCC6* gene maps on chromosome 16 (16p13.1) and encodes multidrug resistance protein-6 (MRP-6), a transmembrane protein involved in transport of molecules between the extra-cellular space and the inside of the cell. The physiological role of MRP6 and its substrate specificity is unclear, although recently multiple sources of evidence indicate involvement in the regulation of human tissue calcification [121, 122]. MRP6 has a widespread tissue distribution but is highly expressed in the basolateral membrane of hepatocytes and proximal kidney tubules [123, 124]. In humans, mutations in *ABCC6* gene cause PXE [125-127] and in some cases, mutations in *ABCC6* were also associated with GACI, a disorder associated with *ENPP1* mutations [96]. The abcc6$^{-/-}$ mouse model was negative for expression of Mrp6 in the liver and has profound mineralization of several tissues including skin, arterial blood vessels and retina [128], which resembles the human PXE phenotype. Furthermore these mice display a 40% reduction in plasma PPi

levels [129]. According to Jansen and collaborators [129], PXE is not caused by a lack of functional MRP6 in the affected tissues, but rather by the absence of PPi that is normally provided to the circulation by an MRP6 mechanism. Recently it has been proposed [130] that polymorphisms in genes known to regulate cellular pyrophosphate metabolism, such as *ALPL*, *ENPP1* and *ANKH*, are genetic risk factors contributing to PXE.

2.4.3 Genetic variants associated

2.4.3.1 Genetic studies on DISH

The variation in the prevalence of DISH throughout the world [2], the existence of familial cases with early onset (in the third decade of life) [64] and the higher frequency of DISH in specific dog breeds [77, 78] suggests that genetic factors might play a part in its aetiology. So far, however, no single gene has been conclusively associated with the disease and very few genetic studies on DISH have been published to date. Some of the first genetic studies in DISH were performed on Major Histocompatibility Complex (MHC) genes (Human Leukocyte Antigens, HLA), due to the similarity of radiographic patterns shared with AS, a spondyloarthropathy with a well-known association with the HLA-B*27 allele [131, 132]. The first study, performed by Shapiro et al [133], reported a positive association between HLA-B27 and DISH, since 16 out of the 47 studied patients were HLA-B27 positive (34%). The authors hypothesized that this gene could be involved in new bone formation due to the association with two disorders such as AS and DISH, where bone proliferation is an essential feature [134]. This study was followed by many more that rejected the hypothesis and the association of HLA alleles and DISH was discarded [135]. Conflicting results were reported in relation to HLA alleles [136-141] and associations of HLA-B8 [142] and HLA-B5 [143] to DISH were proposed but has never been proven. In a relatively small study, 65 individuals affected with DISH and 2352 controls, Vitamin D Receptor (*VDR*), collagen Type $I_{\alpha 1}$ (*COL1A1*) polymorphisms were investigated. These genes are involved in bone density; *COL1A1* is involved in determination of bone density and the interaction of both *COL1A1* and *VDR* with calcium intake regulate changes of bone density over time [144]. Despite the importance of *VDR* and *COL1A1* in bone homeostasis, the authors conclude that neither of these genes seem to contribute to DISH aetiology [145].

Another study investigated the influence of polymorphisms in collagen 6A1 gene (*COL6A1*) in 97 Japanese DISH patients (298 Japanese controls) and 96 Czech DISH

patients (96 Czech controls). One polymorphism (Intron 32; -23) was associated with DISH in Japanese patients but was not associated with DISH in Czech patients. Even so, the authors suggested that *COL6A1* could be responsible for the hyperostotic state leading to ectopic bone formation in spinal ligaments [34]. *COL6A1* encodes an extracellular matrix protein that may serve as a scaffold for osteoblast or pre-osteoblast cells or chondrocytes that subsequently contribute to membranous or endochondral ossification [34]. The *COL6A1* gene is also strongly associated to OPLL [33, 146], and polymorphisms in this gene are considered helpful markers of OPLL disease [6].

A whole genome linkage analysis followed by an "identity-by-state/descent" was performed in DISH/CC Azorean families to clarify the genetic basis of these pathologies in these families, and two zones in chromosome 12 and 20 were found [29]. Another attempt to find genetic associations was undertaken by Jun et all [147], who found that two SNPs (rs1476217 and rs3747676) in the *FGF2* gene were associated with DISH and, one of these (rs1476217), was also associated with OLF. The *FGF2* gene is involved in FGF signalling, which controls bone formation by regulating the expression of various genes involved in osteoblast differentiation and apoptosis [148].

Only two genes have been shown to have a positive association with DISH susceptibility; *COL6A1* [34] and *FGF2* [147] (Table 2-2). However, all the gene variants that showed significant association were located in non-coding regions and were common variants within the general population, which suggests that these variants have a minor effect on DISH susceptibility. In conclusion, the genetic aetiology of DISH is still unknown since the studies that have looked at the possible genetic association with DISH are still inconclusive since none of the studied genes have been shown to be pathogenetically relevant for DISH patients.

Table 2-2. Genetic variants associated with DISH.

Gene	Chr	Physiological function	Study type No Subjects [case/control]	SNP- significantly associated	Ref
COL6A1	**21**	Involved in membranous or endochondral ossification.	Association study [97/298] (Japanese) and [96/96] (Czech) (Assessed 7 SNPs in *COL6A1* gene).	Intron 32 (-29) (only in Japanese) (chi2 = 9.33; **p** = 0.0022), but not with the Czech DISH patients.	[34]
FGF2	**4**	Involved in bone formation by regulating the expression of various genes involved in osteoblast differentiation and apoptosis.	Association study with 154 OPLL, 3 OPLL with DISH and 222 controls.	rs1476217 (**p**=0.03) rs3747676 (**p**=0.01)	[147]

Abbreviations: SNP- Single nucleotide polymorphism, Chr: chromosome, p: p-value, Ref: References.

2.4.3.2 Genetic studies on OPLL

As in many other diseases, the genetic basis of OPLL is now being uncovered with the help of rapidly advancing genome science and technology. The aetiology of OPLL has been extensively investigated from many perspectives and despite the existence of conflicting results, a great number of genetic variants have been associated with susceptibility and severity of OPLL along the years (Table 2-3).

As with DISH, some of the first genetic studies in OPLL were performed on HLA, and this putative association has been extensively discussed in the literature. Koga et al [70] in 1998 provided a genetic linkage study of 91 affected sib pairs and they identified a predisposition locus for OPLL on 6p, close to the HLA complex. Based on this study several other studies were performed in this region [149-151], however the association between HLA alleles and OPLL have not been clarified and this association does not appear to imply causation [152].

Table 2-3. Genetic variants associated with OPLL. The physiological functions of proteins are also indicated (obtained from GeneCards).

Gene	Chr	Physiological function	Study type No Subjects [case/control]	SNP- significantly associated	Ref
IL-1β	2	Stimulates thymocyte proliferation by inducing IL-2 release, B-cell maturation and proliferation and fibroblast growth factor activity.	Case-control association study [120 (43F) /306 (140F)] (Japanese) (Assessed 5 candidate genes; 5 SNPs)	*IL1B AbaI* polymorphism (gender specific – female) (p=0.001)	[153]
ESR1	6	Essential for sexual development and reproductive function, but also play a role in tissues such as bone.		ER (XbaI) gender specific (female) (p=0.007)	
			Large Scale Case-control study [711/896] (Japanese) (Assessed 35 candidate genes; 109 SNPs)	rs9340799 (p=0.017) no correction rs2228480 (p=0.034) no correction	[154]
AHSG	3	Promotes endocytosis, possesses opsonic properties and influences the mineral phase of bone. Shows affinity for calcium and barium ions.		rs2077119 (p=0.0011)	
TGFB3	14	Involved in embryogenesis and cell differentiation.		rs2268624 (p=0.00040 / p=0.044 after Bonferoni Correction) rs2284792 (p=0.037) no correction	
ACE	17	Plays a key role in the renin-angiotensin system.	Case control association study [95/274] (Korean) (Assessed I/D polymorphism in *ACE)*	rs4646994 (genotype DD p<0.001; D allele p=0.009)	[155]
BMP2	20	Induce bone and cartilage formation; member of TGFβ superfamily.	Case control study [192/304] (Assessed 2 SNPs in Exon 3 of *BMP2*)	rs3178250 (p=0.003 gender specific – males)	[156]
			Case control study [57/135] (Assessed 2 SNPs in exon 2 of *BMP2*)	rs2273073 (p<0.001) susceptibility to OPLL rs1049007 (p=<0.001) severity of OPLL	[157]
			Case control study [420/506] (Assessed all coding sequencing of *BMP2*)	rs2273073(p<0.001) rs235768 (p=0.005)	[158]
BMP4	14	Induce bone and cartilage formation.	Nonparametric linkage study with 126 affected sib-pairs using microsatellite markers in 88 candidate genes	Only *BMP4* gene reached criteria of suggestive evidence of linkage (NPL=2.23; p=0.035)	[159]
			Case control association study [179/298] (Chinese). (Assessed 2 polymorphisms in *BMP4*)	rs17563 (genotype: p=0.039; Allele: p=0.014)	[160]
			Case control association study (450/550) (Chinese) (Assessed complete genomic *BMP4* coding)	rs17563 rs76335800	[161]
BMP9	10	Could be involved in bone formation.	Case control association study [450/550] (Chinese) (Assessed complete genomic *BMP9* coding)	rs75024165 (p<0.001) rs34379100 (p<0.001)	[162]
COL11A2	6	Plays an important role in fibrillogenesis. May contribute to the formation of ectopic bone by enhancing endochondral ossification.	Genetic Linkage, association and haplotype analysis study of 91 affected sib pairs from 53 Japanese families	Promoter (-182) (p=0.02); rs1799907 (p=0.0004); rs1799910 (p=0.02); rs1799911 (p=0.03) Haplotypes	[70]
			Haplotype association [161/163]	rs1799907 (p=0.0003) (haplotype with 4 SNPs) male association	[61]
COL17A1	10	Plays a role in the integrity of hemidesmosome and the attachment of basal keratinocytes to the underlying basement membrane.	WES and association studies [28/100] (Chinese)	rs805698 (p=0.00023) rs4918079 (p=0.003)	[163]
PTCH1	9	Functions as a tumor supressor.		p.P1232L; p.T265S	[163]
COL6A1	21	A cell binding protein and may lead to increased bone mass.	Genomewide linkage study followed by fine mapping and haplotype analysis of 142 affected sib pairs	intron 32 (-29) (p=0.000003) rs2236485 (p=0.0002)(MAF 0.13) rs2236486 (P=0.00005)(MAF 0.39) rs2236487 (p=0.00006) (MAF 0.37)	[33, 146]
			Case control association study [90/155] (Chinese)	Promoter (-572) (p=0.000215) Intron 32 (-29) rs2236486 (p=0.00483)	[33]
ENPP1	6	Functions in bone mineralization and soft tissue calcification by regulating pyrophosphate levels.	ttw mouse studies	Gly568stop	[89]
			Association study [323/332] (Assessed all coding sequencing of *ENPP1* gene)	IVS20-11delT (p=0.0029)	[164]
			Case-control association study [180/265]	IVS15-14T --> C (p<0.0001)	[165]
			Association study [95/90] (Chinese) (Assessed 4 SNPs in *ENPP1*)	C973T (p<0.001) IVS15-14T-C (p=0.026)	[166]

Gene	Chr	Physiological function	Study type No Subjects [case/control]	SNP - significantly associated	Ref
HLA	6	Involved in the presentation of foreign antigens to the immune system.	Family based association study with 33 families of patients with OPLL		[151]
			Family based association study with families of 24 patients with OPLL		[150]
IL-15RA	10	Enhance cell proliferation and expression of opoptosis inhibitor.	A case control study [235/250] (Chinese)	rs2228059	[167]
			Association study [166/230] (Korean)	rs2228059	[168]
RUNX2	6	Involved in osteoblastic differentiation and skeletal morphogenesis.	Case control study (Sequenom system) [82/118] (Chinese) (Assessed 19 SNPs in 4 candidate genes)	rs1321075 (p=0.043) rs12333172 (p=0.034)	[35]
RXRB	6	A member of retinoid receptor family regulating a wide variety of biological processes including development, differentiation, and cellular metabolism.	Association study and haplotype analysis [134/158] (Japanese)	3'UTR (+140) (p=0.0028) 3'UTR (+561) (p=0.034)	[169]
TGFB1	19	Mediates bone development and metabolism.	A case control [46/273]	T869-->C	[170]
VDR	12	Plays a central role in calcium homeostasis.	Case-control study [63/126]	VDR FF genotype	[171]
RSPH9	6	Play a role in the membranous ossification process.	Genome Wide association study [1130/7135] (Japanese) followed by an Association study (for replication) [548/6469] (Japanese)	rs927485 ($p=9.4x10^{-9}$)	[172]
STK38L	12	Play a role in the membranous ossification process.		rs11045000 ($p=2.95x10^{-11}$)	
RSPO2	8	Implicated in the endochondral ossification process.		rs374810 ($p=1.88x10^{-13}$) rs13279799 ($p=1.28x10^{-10}$)	
CCDC91	12			rs1979679 ($p=4.34x10^{-12}$)	
HOA1	20	Implicated in the endochondral ossification process.		rs2423294 ($p=1.10x10^{-13}$)	
FGFR1	8	Plays an essential role in the regulation of embryonic development, cell proliferation, differentiation and migration.	Association study [157/222] (Assessed 9 SNPs in 3 genes)	rs13317 (p=0.02)	[147]
BID	22	Has a role in opoptosis signaling.	Association study [157/209] (Korean) (Assessed 2 coding SNPs in *BID*)	rs8190315 (p=0.0052) rs2072392 (p=0.0052)	[173]
TGFBR2	3	Regulate the transcription of a subset of genes related to cell proliferation.	Association study [21/42]	rs11466512 (p=0.007) rs56105708 (p=0.024)	[174]
VKORC1	16	Involved in vitamin K metabolism.	Association study [98/200] (Korean)	rs9923231 (p=0.004) (female)	[175]
IFNG	12	It is a potent activator of macrophages.	Association study [135/222]	rs2430561 rs3138557	[176]

Abbreviations: SNP- Single nucleotide polymorphism, NA: Not applicable, Ref: References, *ACE*: Angiotensin I Converting Enzyme; *BMP2*: Bone Morphogenetic Protein 2; *BMP4*: Bone Morphogenetic Protein 4; *BMP9*: Bone Morphogenetic Protein 9; *COL11A2*: Collagen Type XI Alpha 2; *COL17A1*: Collagen Type XVII Alpha 1; *COL6A1*: Collagen Type VI Alpha 1; *ENPP1*: Ectonucleotide Pyrophosphatase/Phosphodiesterase 1; *ESR1*: Estrogen Receptor 1; *HLA*: Human Leukocyte antigen; *IL-15RA*: Interleukin 15 Receptor Alpha; *IL-1β*: Interleukin 1 Beta; *PTCH1*: Patched 1; *RUNX2*: Run-Related Transcription Factor 2; *RXRB*: Retinoid X Receptor Beta; *TGFβ1*: Transforming Growth factor Beta 1; *TGFβ3*: Transforming Growth factor Beta 3; *AHSG*: Alpha 2-Heremans-Schmid glycoprotein; *VDR:* Vitamin D Receptor; *RSPH9:* radial spoke head 9 homolog; *STK38L:* serine/threonine kinase 38 like; *RSPO2:* R-spontin 2; *CCDC91:* Coiled-coil domain containing 91 ; *HOA1:* Hydroxyacid oxidase 1; *FGFR1:* Fibroblast Growth Factor Receptor 1; *BID*: BH3 Interacting Domain Death Agonist. *TGFBR2:* Transforming Growth Factor, Beta Receptor II. *VKORC1*: Vitamin K epoxide reductase complex subunit 1; *IFNG*: Interferon, Gamma.

Along the years several other genes located on chromosome 6 have been investigated. *COL11A2*, located at 6p21.3 was analyzed by Koga et al [70] for the presence of molecular variants associated with OPLL and 4 variants (promoter -182, rs1799907, rs1799910 and rs1799911) showed strong statistical association with OPLL (Table 2-3). On the other hand, according to Maeda [177] the rs1799907 polymorphism, previously associated with OPLL [70], might act as a protective allele in the development of OPLL, since the polymorphism is more frequently observed in controls than in OPLL patients. Interestingly, the authors found that rs1799907 resulted in altered splicing in the region containing exons 6 through 8 with preservation of exon 7. The protective effect is proposed to be due to the higher frequency of the rs1799907 allele in white populations, in whom a low frequency of OPLL has been reported. Maeda et al [61] in another study found a male-specific association of a *COL11A2* haplotype with OPLL. Other genes located in chromosome 6 such as RXRβ [169], *ESR1*, *RSPH9* and *RUNX2* were also investigated and in all of them SNPs associated with OPLL were identified (Table 2-3).

Based on the initial findings, that a nonsense mutation in the *Npps* gene (*ENPP1*; MIM*173335) in the naturally occurring mutant ttw mouse causes ectopic ossification of the spinal ligaments that resembles OPLL [89], several studies have tried to associate this gene to OPLL. ENPP1 is the main enzyme that generates PPi (a known inhibitor of calcification), in osteoblasts and chondrocytes, regulating bone mineralization by decreasing hydroxyapatite crystal deposition [178]. Nakamura et al [164] in an association study found that *ENPP1* is involved in OPLL by revealing an allelic association with an intron 20 polymorphism (denoted as IVS20-11delT). This polymorphism was significantly higher in OPLL patients than in controls, indicating that individuals with this variation may be more susceptible to abnormal ossification of the spinal ligaments. This variant is considered a risk factor for diabetes mellitus type 2 and obesity [179]. In another study the same variant - IVS20-11delT- in *ENPP1* and the A861G variant of the leptin receptor gene were more frequent in patients with OPLL in the thoracic spine relative to those with OPLL restricted to the cervical spine, leading the authors to suggest that both variants are associated with more extensive OPLL, but not with the frequency with which it occurs [180]. Other studies, however, reported that IVS20-11delT was unassociated with OPLL [154, 165] and other associated variants such as IVS15-14T [165] was associated with susceptibility and severity of OPLL. Other

authors showed that the polymorphism TT genotype of C973T and IVS15-14T in the same gene were associated with more severe disease [166].

In addition, *COL11A2*, *COL6A1* that map to chromosome 21 and *COL17A1* that maps to chromosome 10 have also been studied in OPLL susceptibility. The *COL6A1* gene [33, 146, 181], is strongly associated with OPLL and polymorphisms of this gene are considered helpful markers of OPLL disease. However, this association has not been confirmed by some authors [35, 72, 159]. Polymorphisms of this gene are also related to DISH in a Japanese population [34] and this suggests that *COL6A1* may not only play a role in OPLL, but in pathological ectopic ossification in general. A whole exome sequencing study revealed that *COL17A1* was associated with OPLL and two SNPs in this gene rs805698 and rs4918079 were significantly associated with OPLL [163].

Bone morphogenic proteins (*BMP2, BMP4* and *BMP9*) and TGFβ superfamily proteins also play a role in OPLL pathogenesis. A positive association to OPLL was found with the following SNPs: rs3178250 [156], rs2273073 [157, 158] and rs1949007 [157] in the *BMP2* gene. Furthermore Yan in 2013 [158], showed that the rs2273073 variant in the *BMP2* gene was positively associated with the level of Smad4 protein expression and the activity of alkaline phosphatase. Activation of Smads causes their translocation from the cell cytoplasm to the nucleus where they control gene expression [182], and alkaline phosphatase is the first functional gene expressed in the process of calcification and is secreted by osteoblasts in the process of osteogenic differentiation [183]. On the other hand, Kim et al [72] showed that SNPs rs2273073 and rs1949007 in *BMP2* gene may not influence the OPLL in Korean patients. Other study elaborated by Liu et al [35] also failed to show the association of *BMP2* gene to OPLL in the Chinese Han population. The BMP4 protein is another candidate gene for OPLL and two SNPs rs17563 [160, 161], rs76335800 and a specific haplotype, TGGGCTT [161], were demonstrated to contribute to the risk of developing OPLL in a Chinese population. The association of *BMP-4* with OPLL was also confirmed by Furushima et al [159] in a large scale screening study for candidate genes in OPLL, in which only the *BMP-4* gene reached the criteria for suggestive evidence of linkage. In the *BMP9* gene two SNPs have been associated with OPLL: rs75024165 and rs34379100 [162].

Another interesting gene with conflicting results is *TGFβ1*, that, *a*ccording to Kamiya et al [170], is genetically determinant in predisposition for the condition (869T>C; rs1982073). However Han et al [184] showed that the SNP previously associated with OPLL (869T>C; rs1982073) and the promoter (-509C>T; rs1800469) were not

associated with OPLL in a Korean population. Similarly, in one study that assessed 109 polymorphisms of 35 candidate genes in 711 patients with OPLL and 896 controls failed to confirm the positive association of OPLL with *TGFβ1* gene. Interestingly, in the ossified matrix and chondrocytes of adjacent cartilaginous areas of OPLL, TGF-beta1 is overexpressed, and one study examined if there is an association between the SNP 869T>C; rs1982073 and the radiological appearance of OPLL. Although a T-C transition in TGF-beta1 did not predict a difference in the radiographic appearance of the ossified segment of posterior longitudinal ligament, it was associated with the location of OPLL within the spinal column. According to the authors *TGFβ1* polymorphisms are not related to the onset of OPLL, but rather to the area of the ossified lesion. The "C" allele might be a risk factor for patients with OPLL in other areas in addition to the cervical lesion [185]. Association of *TGFβ3*, which plays an important role in regulating chondrogenic differentiation of mesenchymal stem cells in humans [186] has also been described [154] and an intronic SNP rs2268624 has a significant association with OPLL.

Other genes from large scale studies have recently emerged as promising targets for future investigation, including *AHSG*, *STK38L*, *RSPO2*, *CCDC91* and *HOA1*. In a Genome wide linkage study [187] with 214 affected sib-pairs the D20S894 marker on chromosome 20p12 was associated with OPLL. The linkage region contained 25 known genes and two genes located in this linkage region may be good candidate genes for OPLL, one example is Jagged 1 (*JAG1*) because of its potential involvement in the endochondral bone formation [188] and *BMP2* already studied and an already established important regulator of bone metabolism. Very recently 8 potentially pathogenic missense variants in 4 genes, including 3 in *COL6A1*, 2 in *COL11A2*, 2 in *FGFR1* and 1 in *BMP-2* were identified in a target exome sequencing study with 11 OPLL candidate genes using 55 Chinese patients with OPLL [181]. Although the authors suggested that these missense variants were involved in pathogenicity of OPLL, further confirmation is required.

A large number of genes have already been associated with OPLL, nonetheless it is probable that many other potential genes are involved in inheritance of the disease. OPLL does not appear to follow a simple, single gene Mendelian inheritance pattern and it is most likely multifactorial and develops in individuals with a genetic predisposition due to a variety of different mutations in various genes on various chromosomes.

2.4.4 Genetic studies on OLF

As far as we known, there are few studies in the literature that investigate a disease causing gene in OLF. Kong and collaborators [33] investigated the frequency of 4 SNPs in *COL6A1* gene, a well-known OPLL susceptibility gene for OLF and OPLL, in 183 patients (61 with OLF) and 155 controls and suggest that *COL6A1* may be a common susceptibility gene for OLF in Chinese population (Table 2-4). Another study [35] investigates the possible association of four genes, that may be related to ossification of spinal ligaments (*RUNX2*; *BMP2*; *COL6A1* and *VDR*), and the authors found that *RUNX2* (rs1321075 and rs12333172 polymorphisms) could be responsible for ectopic bone formation in the spinal ligament in a Chinese population. Jun et all [147] investigated the association between *FGF2* (Fibroblast Growth Factor), *FGFR1* (Fibroblast Growth Factor Receptor 1) and *FGFR2* (Fibroblast Growth Factor Receptor 2) polymorphisms with OLF and OPLL and found a positive association of the SNP rs1476217 in *FGF2* gene (Table 2-4).

Table 2-4. Genetic variants associated with OLF.

Gene	Chr	Physiological function	Study type No Subjects [case/control]	SNP-significantly associated	Ref
COL6A1	21	Collagen VI is a major structural component of microfibrils. Mutations in the genes that code for the collagen VI subunits result in the autosomal dominant disorder, Bethlem myopathy.	Case control association study with 61 OLF patients and 155 Chinese controls. Assessed 4 SNPs in *COL6A1*	rs17551710 (p=0.0005)	[33]
RUNX2	6	*RUNX2* is a transcription factor that encodes a nuclear protein with a Runt DNA-binding domain. This protein is essential for osteoblastic differentiation and skeletal morphogenesis and acts as a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression. Mutations in this gene have been associated with the bone development disorder cleidocranial dysplasia.	Case control association study with 36 Chinese patients (12 with OLF and 22 with OLF and OPLL) and 118 controls	rs1321075 (p=0.034) rs12333172 (p=0.043)	[35]
FGF2	4	The protein encoded by this gene is a member of the FGF family. This protein has been implicated in diverse biological processes, including limb and nervous system development.	Case control association study of 157 OPLL patients (29 with OLF) and 222 controls	rs1476217 (p=0.01)	[147]

Abbreviations: SNP: Single nucleotide polymorphism, Chr: chromosome, p: p-value, Ref: references, *COL6A1*: Collagen Type VI Alpha 1 Chain, *FGF2*: fibroblast growth factor 2, *RUNX2*: Runt-related transcription factor 2.

2.5 Association with other diseases

The presence of OSL has been reported in association with numerous disorders of different etiologies.

2.5.1 Monogenic disorders

Case reports of patients with monogenic metabolic disorders with co-occurrence of both DISH and OPLL have been reported in literature (Table 2-5). All of the disorders presented in table 2-5 are directly involved in calcium and phosphate homeostasis, with the exception of alkaptonuria, a disorder of tyrosine metabolism characterized by the accumulation of homogentisic acid, ochronosis, and destruction of connective tissue resulting in degenerative spondylosis and arthritis [189]. In ochronosis, calcification of intervertebral disks due to calcium hydroxyapatite and marked sclerosis including sacroiliac joint can also be observed. Genes involved in Hypophosphatemic rickets and Hypophosphatasia are, as expected, directly involved in phosphate homeostasis. Analysing the genes present in table 2-5 the *FGF23* gene inhibits phosphate uptake and mineralization in vivo by suppressing expression of type IIc (*SLC34A1*) sodium/phosphate cotransporters in the brush border membranes of proximal tubes [190]. *SLC34A1* contributes to the maintenance of inorganic phosphate concentration in the kidney [191]. *DMP1* gene encodes the Dentin matrix acidic phosphoprotein 1, which plays a role in controlling osteocyte formation and phosphate homeostasis [192]. Proteolytic degradation of DMP1 leads to the release of the "Acidic serine- and aspartate-rich MEPE-associated motif" (ASARM) peptides, which are potent inhibitors of mineralization (minhibins) [193]. The ASARM peptide inhibits mineralization directly by binding to hydroxyapatite crystals and decreasing expression of *PHEX*, whereas phosphaturic effects are mediated by renal accumulation of ASARM peptides and inhibition of Pi reabsorption. *PHEX*, a Type II, zinc dependent, transmembrane endopeptidase is involved in bone and dentin mineralization as well as in renal phosphate reabsorption [14]. *CLCN5* is a renal chloride channel gene highly expressed in proximal tubules of the kidney that play an important role in renal tubular function [194].

Table 2-5. Monogenic disorders previously associated with OSL, type of inheritance, number of Mendelian Inheritance in Man database (MIM) and gene involved. Lack of inheritance means that it is unknown.

Disorder	Inheritance	OMIM	Gene/Locus Involved	Ref
Hypophosphatemic rickets/osteomalacia	AD	193100	*FGF23*	[195]
	AR	241520	*DMP1*	
	AR	613312	*ENPP1*	
	AR	241530	*SLC34A3*	
	XLD	307800	*PHEX*	
	XLR	300554	*CLCN5*	
Hypophosphatasia	AR	241500	*ALPL*	[196]
	AR	241510		
	AR, AD	146300		
Pseudohypoparathyroidism	AD	103580	*GNAS1*	[197]
Hypoparathyroidism	AD	146200	*GCM2*	[198-200]
	AD/AR	146200	*PTH*	
Alkaptonuria	AR	203500	*HGD*	[201]
Acromegaly	Somatic /AD	102200	*AIP*	[202]
		102200	*GNAS1*	
	X linked	300943	*GPR101*	
	AD	610755	*CDKN1B*	
	AD	131100	*MEN1*	
	Somatic	174800	*GNAS*	
	AD	160980	*PRKAR1A*	
Familial Hypocalciuric Hypercalcemia	AD	145980	*CASR*	[203]
	AD	145981	*GNA11*	
	AD	600740	*AP2S1*	

Abbreviations: AD - Autosomal Dominant, AR - Autosomal Recessive, XLD - X-linked Dominant and XLR X-linked Recessive, Ref: References, *ALPL*: Alkaline Phosphatase, Liver/Bone/Kidney, *FGF23*: Fibroblast growth factor 23, *DMP1*: Dentin matrix acidic phosphoprotein 1, *ENPP1*: ectonucleotide pyrophosphatase/phosphodiesterase 1, *SLC34A3*: Solute carrier family 34 member 3, *PHEX*: Phosphate regulating endopeptidase homolog, X-linked, *CLCN5*: Chloride voltage-gated channel 5, *GNAS1*: Guanine nucleotide binding protein, alpha stimulating, *GCM2*: Glial cells missing Homolog 2, *PTH*: Parathyroid hormone, *HGD*: Homogentisate 1,2-Dioxygenase, *AIP*: Aryl hydrocarbon receptor-interacting protein, *GPR101*: G protein-coupled receptor 101, *CDKN1β*: Cyclin-dependent kinase inhibitor 1β, *MEN1*: Menin 1, *PRKAR1A*: Protein Kinase CAMP-Dependent Type I Regulatory Subunit Alpha, *CASR*: calcium-sensing receptor, *GNA11*: G Protein Subunit Alpha 11, *AP2S1*: Adaptor Related Protein Complex 2 Sigma 1 Subunit.

Alkaline phosphatase (*ALPL*) is present in matrix vesicles and has the ability to hydrolyze PPi, playing a role in bone mineralization. In hypophosphatasia the *ALPL* mutations leading to deficient activity of the tissue-non-specific alkaline phosphatase isozyme (*TNAP*), an enzyme which converts the inhibitor (PPi) into a promoter of mineralization

(Pi) [183]. Lastly, but not least is *ENPP1* gene, which has been already mentioned previously. It is particularly interesting to see that the only report of an OPLL patient with hypophosphatemic rickets is caused by a novel homozygous mutation in the *ENPP1* gene [195]. However, not all patient cases of Hypophosphatemic Rickets caused by mutations in *ENPP1* present OPLL.

Hypoparathyroidism cases associated with changes resembling DISH are reported in the literature [198-200]. The genes involved in Hypoparathyroidism *GNAS*, *GCM2* and *PTH* are also involved in both calcium and phosphate metabolism. *GNAS* (GNAS Complex Locus) play a role in signaling pathways that regulate osteogenesis, normally preventing ectopic ossification in tissues where bone should not form [204]. *GNAS* mutations have been associated with several other disorders, including Progressive Osseous Heteroplasia (POH; MIM#166350). *GCM2* (Glial Cells Missing Homolog 2) gene encodes a protein that possibly is involved in a backup endocrine mechanism for the regulation of calcium homeostasis in the absence of parathyroid glands [205]. The *PTH* gene encodes parathyroid hormone that is secreted when calcium levels drop and causes increased calcium absorption through the gut, and kidney and provokes an increase in bone resorption through direct and indirect processes. It is an essential hormone in bone homeostasis [206].

Acromegaly is a rare condition characterized by excess growth hormone (GH) production by the pituitary gland and its principle mediator insulin-like growth factor 1 (*IGF-1*) [207]. Familial syndromes associated with GH hypersecretion can be seen in table 2-5. Excess GH and IGF-1 cause proliferation of articular chondrocytes and increased matrix production [208]. It is known that GH levels may act as a bone promoting factor in DISH [209].

There is only one case report of a patient with Familial Hypocalciuric Hypercalcemia (FHH), a rare and benign cause of lifelong hypercalcemia, and DISH. This particular case, a 45-year-old diabetic woman with hypercalcemia secondary to FHH, developed dysphagia because of external esophageal compression of DISH [210]. A causal association between FHH and DISH is not yet proven.

2.5.2 Complex disorders

Some authors suggest that DISH, instead of a disorder by itself is only a clinical expression of metabolic disorders being angiogenesis the link leading to new bone formation [211]. The aetiology of the following associated OSL disorders is complex and

determined by the interplay of genetic and environmental factors. Environmental factors such as age, smoking, alcohol, diet, and physical inactivity may directly influence Type 2 Diabetes mellitus (T2D), metabolic syndrome and obesity. Nevertheless, although heterogeneous, there are some monogenic forms of these disorders; see table 2-6 for more details.

Table 2-6. Complex disorders previously associated with OSL. Lack of inheritance means that it is not yet known.

Disorder	Type	Inheritance	OMIM	Gene/Locus Involved	Ref
Non-insulin-dependent Type 2 Diabetes mellitus	Monogenic - MODY	AD	606391	Genetically Heterogeneous – associated with mutations in 13 genes	[212, 213]
	Polygenic	?	125853	Many susceptibility locus identified, including in *ENPP1*	
Abdominal Obesity - Metabolic Syndrome	Monogenic	?	615812	*DYRK1β*	[214]
		AR	200100	*MTP*	
	Polygenic	?	605552	*AOMS1 locus* *AOMS2 locus*	
Obesity	Monogenic	AR	614962	*LEP*	[215]
		AR	614963	*LEPR*	
		AR	600955	*PCSK1*	
		AR	609734	*POMC*	
	Polygenic	?	601665	Genetically heterogeneous but including *ENPP1* as susceptibility gene	

Abbreviations: OMIM: Online Mendelian Inheritance in man, MODY: Maturity-Onset Diabetes of the Young, AD: Autosomal Dominant, AR: Autosomal Recessive, Ref: References, *ENPP1*: ectonucleotide pyrophosphatase/phosphodiesterase 1, *DYRK1B*: Dual specificity Tyrosine Phosphorylation regulated kinase 1β, *MTP*: Microsomal triglyceride transfer protein, *AOMS1,2*: Abdominal obesity-metabolic syndrome 1,2, *LEP*: Leptin, *LEPR*: Leptin receptor, *PCSK1*: Proprotein convertase subtilisin/kexin 1, *POMC*: Pro-opiomelanocortin.

Diabetes mellitus is considered to be a heterogeneous group of disorders, which are characterized by persistent hyperglycemia. There are rare forms of diabetes that are monogenic, as is the case of maturity onset diabetes in the young (MODY; 606391),

however the two most common forms of diabetes are T1D, also known as insulin-dependent diabetes and T2D, known as non-insulin-dependent diabetes. Both T1D and T2D are polygenic, caused by a combination of genetic and environmental risk factors. Individuals with T2D usually have a tendency for having obesity and manifestations of the metabolic syndrome, which is characterized by diabetes, insulin resistance, hypertension, and hypertriglyceridemia [216]. T2D can be associated with OSL by either hyperglycemia or by the high insulin rate [217]. Studies already performed are not conclusive. Although obesity is considered to be a complex and multifactorial disease, there are some monogenic cases described as rare and severe early-onset obesity associated with endocrine disorders. The phenotype, in monogenic cases, is due to mutations in genes of the leptin/melanocortin axis involved in food uptake regulation - genes of leptin (*LEP*) and its receptor (*LEPR*), Pro-opiomelanocortin (*POMC*) and proprotein convertase subtilisin/kexin 1 (*PCSK1*) [218]. Mutations in the *LEPR* gene, occurs in the ZFR rat model and can cause obesity, hyperinsulinemia, hypercholesterolemia and hyperlipidemia besides the ossification of spinal ligaments, resembling human OPLL [42]. It is also relevant to see *ENPP1* as a susceptibility gene for both T2D [219] and obesity [220].

2.5.3 Rheumatic disorders co-existing with OSL

The co-existence of DISH with rheumatic disorders has been described since its first report by Forestier and Rotes Querol, in 1950 [221] and a high percentage of DISH cases are complicated by OPLL [6] suggesting that they share common aetiopathogenic factors. Simultaneous OPLL and OLF are also very common in the literature [222, 223]. Additionally, the co-existence of the three OSL disorders – DISH; OPLL and OLF has also been reported [32].

The genetic basis of coexistent rheumatic conditions with OSL can be seen in table 2-7.

Table 2-7. Rheumatic disorders previously seen coexisting with OSL. Lack of inheritance means that it is not yet known.

Disorder	Inheritance	OMIM	Gene/Locus Involved	Ref
Ankylosing Spondylitis (AS)	Multifactorial	106300	*HLA-B*	
	AD	183840	*SPDA2 locus*	
		613238	*SPDA3 locus*	
Chondrocalcinosis	AD	118600	*ANKH*	[224, 225]
	AD	600668	*CCAL1 locus*	
Rheumatoid arthritis	?	180300	*6q23 (HLA)*	[22, 226-228]
	?	604302	*IL6/MIF*	
Gout	AD;AR	612076	*SLC2A9*	[22, 67, 229, 230]
	AD	612671	*SLC17A3*	
	AD?	138900	*ABCG2*	
Osteoarthritis	Multifactorial	165720	*FRZB*	[22]
	AD	140600	*MATN3* (with Heberden Nodes)	
	AD	607850	*ASPN*	
	?	612400	*GDF5*	
	AD	604864	*COL2A1*	
	?	610839	*2q33.3*	
	?	612401	*3p24.3*	
Paget's Disease	AD	602080	*TNFRSF11A*	[224, 225]
	AD	167250	*SQSTM1*	
	AD	606263	*PDB4*	
	AR	239000	*TNFRSF11B*	
	AD	616833	*ZNF687*	

Abbreviations: AD: Autosomal Dominant, AR: Autosomal Recessive, Ref: References, ?: unknown, HLA-B: Human Leukocyte antigen B, *SPDA2,3*: Spondyloarthropathy, Susceptibility To, 2,3, *ANKH*: progressive ankyloses protein homolog, CCAL1: Chondrocalcinosis 1, *IL-6*- Interleukin-6, *MIF*: Macrophage inhibitory factor, *SLC2A9*- Solute carrier family 2 member 9, *SLC17A3*- Solute carrier family 17 member 3, *ABCG2*: ATP Binding Cassette Subfamily G Member 2, *FRZB*- Frizzled-related protein 1, *MATN3*: Matrilin-3, *ASPN*- Asporin, *GDF5*- Growth/differentiation factor 5, *COL2A1*- Collagen Type II Alpha 1 Chain, *TNFRSF11A, B*: TNF Receptor Superfamily Member 11a,b, *SQSTM1*: Sequestosome-1, *PDB4*: Paget disease of bone 4, *ZNF687*- Zinc Finger Protein 687.

The co-existence of DISH and **AS** is possible and has been thoroughly described in more recent years due to the difficulty, sometimes, to distinguish them apart [7-21]. OPLL has also been reported in patients with AS but it was determined that OPLL in AS was associated with older age [231]. AS is a chronic, multisystem inflammatory disorder characterized by inflammation and ankylosis of the sacroiliac joints and the axial

skeleton. Structural damage in AS is dominated by new bone formation that can result in spinal fusion and marked functional limitation. In Online Mendelian Inheritance in Man database (OMIM), which collects known genetic lesions responsible for human inherited diseases, the following principal loci of AS susceptibility are mentioned: HLA-B locus (SPDA1; MIM106300), 9q31-q34 (SPDA2; MIM 183840) and 2q (SPDA3; MIM 613238) (Table 2-7), however more than 100 genetic influences are reported in the literature [232] [233, 234], although many of them are pending confirmation. It is known that AS is a polygenic disorder and its heritability is estimated to be more than 90% and the MHC-1 region, particularly the HLA-B*27, is the main contributor for this proportion [235, 236], followed by a second gene definitively associated with AS, the *ERAP1*. Both contribute to MHC1 antigen processing pathway, and the role played by HLA-B27 in AS pathogenesis could be explained by the association between both, and actually this association is considered the two most powerful disease risk factors to AS. *ERAP1* plays a role in trimming peptides within endoplasmic reticulum (ER) into the right length binding to MHC-I molecules, and so abnormal function of ERAP1 can lead to generation of abnormal peptides of incorrect length and sequence, which subsequent to *HLA-B27* binding can lead to peptide-HLA-B27 complex misfolding. These misfolded proteins could either accumulate within ER, thus contributing to the ER stress, or be transported to the cell surface as a free heavy chain. This mechanism seems to have a role in AS pathogenesis, but the degree of this contribution to the overall pathogenesis of AS remains hypothetical. The association of *ERAP1* is restricted to *HLA-B27* positive cases or *HLA-B27* negative/*HLA-B40* positive cases [232, 237, 238]. Several gain of function variants are reported as associated with AS and loss of function as protective to AS [239, 240]. In SPDA3 locus the *IL-1* gene seems to be associated with AS [241]. Another genetic contribution is the involvement of interleukin IL-23/IL-17 pathway, that has been shown to have significance in the pathogenesis of AS. The *IL-23* drives the differentiation of CD4-positive Th17 cells, which produce IL-17. Il-17 in turn facilitate the production of IL-6, IL-8, TNF, chemokines, matrix metalloproteinases, and receptor activator of nuclear factor kβ ligant from a wide range of cell types [242]. Based on genome wide association and linkage studies several risk genes involved in ectopic calcification in AS were reported in the literature. The *ANTXR2* (Anthrax toxin-receptor 2), was identified as one of the risk loci for AS [243]. This gene potentially affects new bone formation as it can interact with LRP6 protein, an important surface receptor in the Wnt/β-catenin pathway. In Han Chinese population the *ANTXR2* gene is not associated with AS [244]. Other two

risks loci that seems to be relevant to bone formation in AS were also reported in the literature, the *HAPLN1-EDIL3* at 5q14.3 and *ANO6* at 12q12.1 [244]. *HAPLN1* is involved in osteophyte formation and disc generation in Japanese women with spinal osteoarthritis [245], and *EDIL3* has an inhibitory effect on WNT/β.catenin signaling [246]. The *ANO6* gene controls bone mineralization by activating the calcium transporter NCX1. In mice, the lack of *Ano6* is responsible to bone mineralization defects [247]. Another relevant gene in mineralization is *ANKH* (human homolog of progressive ankyloses), a gene that is also reported as associated with AS. Curiously this association is gender specific, in other words some SNPs of the *ANKH* gene are significantly associated with AS in men and other SNPs are present in females [248]. ANKH protein regulates PPi export from the cells. It is well known that abnormal PPi levels can be associated with aberrant bone formation and in the mutant mice (ank/ank**),** which have a premature stop codon in the 3' end of the ank gene, severe ankylosis occurs [106].

The co-coexistence of some form of spinal ossification with **chondrocalcinosis** are reported in the literature [25, 26], suggesting that a genetic link exists between these diseases. The first evidence of a genetic link between OPLL and chondrocalcinosis was identified in the ttw mouse a model for OPLL which develops spinal ossification and hydroxyapatite arthropathy. *Npps*, also designated as *PC-1*, is the mutant gene that causes this mouse phenotype. In humans this gene, designated *ENPP1*, plays a role in regulating PPi levels thus regulating bone mineralization and soft tissue calcification [249]. Variants in *ENPP1* are associated with GACI, diabetes (125853), obesity (601665) [250] and other diseases. The possible association of the *ENPP1* gene with OPLL is not yet proven however several studies confirm a positive association (Table 2-3), the association with CC is considered a minor determinant for the disease [251, 252]. DISH and CC is very common in Terceira Island and this has led to the suggestion that both diseases share the same pathogenic mechanism [24]. The coexistence of CC and AS is also reported [253] and CC has also been associated with Gitelman syndrome (GS), a kidney disorder that causes an imbalance of potassium, magnesium and calcium ions, possibly due to their association with chronic hypomagnesaemia [254]. CC, also known as calcium pyrophosphate dehydrate (CPPD) disease, pseudogout or pyrophosphate arthropathy [255] is a disorder of ectopic calcification characterized by the deposition of calcium containing crystals in articular cartilage, synovial membranes and sometimes in periarticular soft tissues. The deposited salts are composed of CPPD although other calcium salts can also be found including hydroxyapatite [256]. In some cases, the

deposition of calcium crystals - hydroxyapatite or CPPD- can also occur in the spinal ligaments [257, 258], but it is usually difficult to distinguish from ossification [46]. The physicochemical mechanisms that cause this deposition are still unknown, but disordered PPi metabolism is suspected. The deposited crystal may be calcium hydroxyapatite (in which the local PPi concentration is presumed to be low) or CPPD (when PPi is high) [255]. In addition to the sporadic form, chondrocalcinosis a hereditary form also occurs and it may also be associated with metabolic disorders - hyperparathyroidism [259], hemochromatosis [260], and hypomagnesemia [261]. In 1963, Zitnan and Sitaj [262], reported a hereditary form of "chondrocalcinosis articularis" in seven Czechoslovakian kindreds. Since then, familial aggregation has been reported in different ethnic groups most of them showing an autosomal dominant inheritance [25, 263-265]. Genetic linkage studies have mapped the familial forms of CC to two particular chromosome regions, CCAL1 (MIM 600668) on chromosome 8 [266] and CCAL2 (MIM 118600) on chromosome 5 [267]. The later contains the human homologue (*ANKH*) of the murine *ank* gene which is expressed in cartilage and other tissues and is associated with CC and spinal and peripheral joint ankylosis in the ank/ank mouse [106]. The *ANKH* gene maps to chromosome 5 (5p15.1) and encodes a transmembrane inorganic pyrophosphate transporter ANK that transports intracellular pyrophosphate into the extracellular matrix, where it acts as a potent inhibitor of mineralization [108]. Analysis of this gene has identified a variety of mutations that segregate with the CC phenotype [110] [268] [29] [30] [31]. These mutations enhance ANK protein activity, thereby elevating extracellular pyrophosphate levels and promoting the formation of pyrophosphate crystals, which produce the manifestations of the disease [269]. For the moment, *ANKH* (CCAL2) is the only monogenic cause identified for CC. In humans, dysfunction of the *ANKH* gene also causes autosomal dominant craniometaphyseal dysplasia (OMIM #123000) [109]. The disease-causing gene in CCAL1 has never been identified but there is a strong possibility that this gene is primarily related to osteoarthritis (OA) and that the CPPD deposition is secondarily, enhanced by the degenerative changes in cartilage [266].

The coexistence of **Rheumatoid arthritis** (RA) with DISH, OPLL and OLF is mentioned in the literature. RA is an inflammatory disease, primarily of the joints, with autoimmune features and a complex genetic component involving many different pathways within the innate and the adaptative immune system [270]. Among the genetic components, genes in the MHC region on chromosome 6 are widely acknowledged as major players in RA. *MIF* (Macrophage migration inhibitory factor) gene also seems to play a role in RA

pathogenesis, the gene encodes a lymphokine involved in cell-mediated immunity, immunoregulation, and inflammation. Other genes that are involved in the disease is Interleukin-6 (*IL-6*) which encodes a cytokine that functions in inflammation and the maturation of B cells.

Gout is a form of arthritis caused by impaired urate metabolism, leading to high serum urate levels (hyperuricemia) and accumulation of sodium urate crystals in the joints.

At least three genes are associated with gout - *SLC17A3*, *SLC2A9* and *ABCG2* - all of them correspond to urate transporters located in the epithelial cells of renal proximal tubules (Table 2-7). The genes most strongly linked to gout corresponds to the solute carrier 2A9 (*SLC2A9*), also known as glucose transporter 9 (*GLUT9*). This transporter was initially identified as a glucose/fructose transporter [271], however it is also a high-affinity urate transporter and functions in renal urate reabsorption. The second locus linked to gout corresponds to the gene encoding the solute carrier 17A3 (*SLC17A3*), also known as the renal sodium phosphate transport protein 3 (*NPT3*), that excretes intracellular urate and organic anions. Mutations in the *SLC17A3* gene caused reduced urate efflux compared to the wildtype when expressed in xenopus oocytes [273].The last gene linked to gout corresponds to *ABCG2*, a gene which encodes a multidrug resistance transporter belonging to the ATP-binding cassette (ABC) superfamily. It has recently been discovered that this transporter is also involved in renal urate elimination, and the presence of a specific polymorphism (Q141K) induces a decrease in urate efflux [274, 275].

Osteoarthritis (OA) is a degenerative disease of the joints characterized by loss and/or remodeling of joint synovium, cartilage, and bone. The sites most frequently affected are knee, hip, feet and hands, although involvement of lumbar and cervical spine, elbows, wrists and other joints can also occur [276, 277]. According to the OMIM database, osteoarthritis susceptibility (OS) has been associated with several regions/genes: *FRZB* (OS1; 165720), *MATN3* (OS2; 140600), *ASPN* (OS3; 607850), region 2q33 (OS4; 610839), *GDF5* (OS5; 612400) and region 3p24 (OS6; 612401). Another susceptibility gene for osteoarthritis (with chondrodysplasia) (304864) is the *COL2A1* gene (Table 2-7). Particularly, *FRZB* (Frizzled-Related protein 1) gene encodes a secreted protein antagonist of wingless (WNT) signaling involved in the regulation of bone development. It is known that functional polymorphisms within *FRZB* causes susceptibility for hip OA in females [278], implicating the WNT signaling pathway in the pathogenesis of this disease. The *MATN3* (Matrilin 3) gene encodes a protein present in the cartilage

extracellular matrix and has a role in the development and homeostasis of cartilage and bone [279]. This gene has recently been shown to bind cartilage extracellular matrix proteins, including collagen types II and IX [280], and promotes chondrogenesis through an interleukin-1 receptor antagonist-dependent mechanism [280, 281]. Variants in this gene result in hand osteoarthritis [277, 282] and other skeletal diseases, including multiple epiphyseal dysplasia, which is characterized by abnormal ossification in the growth plate and early onset OA [283]. *ASPN* is another osteoarthritis susceptibility gene, which encodes asporin, a cartilage extracellular protein that regulates chondrogenesis by inhibiting transforming growth factor-beta 1-induced gene expression in cartilage. In addition, this protein also binds calcium and collagen and may induce collagen mineralization. Polymorphisms in this gene are associated with knee osteoarthritis susceptibility [284, 285]. Associations between this gene and lumbar disk degeneration [286] and AS are also reported [287]. The *GDF5* (Growth Differentiation Factor 5) gene is a member of the TGF-β/BMP superfamily which is involved in bone and cartilage formation by inducing chondroblastic and osteoblastic differentiation and the formation of joints [288, 289]. Mutations in this gene seems to be involved in hip and knee OA progression [290] and other skeletal disorders, such as Acromesomelic dysplasia (AMDH; MIM #201250) [291], Chondrodysplasia (MIM; 200700) [292], among others. The other gene associated with OA is *COL2A1* (Collagen Type II Alpha 1 Chain) which encodes Type II collagen, the major collagen synthesized by chondrocytes. Besides the association of Osteoarthritis with mild chondrodysplasia (604864) [289, 293], this gene is also associated with achondrogenesis (MIM; 200610), Spondyloperipheral dysplasia (MIM; 271700), and other diseases. Lastly but not least, two candidate genes for osteoarthritis susceptibility were found in the region 2q33.3, the Parathyroid Hormone 2 Receptor (*PTHR2*) and Frizzled Class Receptor 5 (*FZD5*), however only a missense variant in the *PTHR2* gene co-segregate with the disease [294]. For the region 3p24.3, several variants in collagen Type VI, alpha-4, pseudogene 1 (*COL6A4P1* or *DVWA*) seem to contribute strongly to knee osteoarthritis susceptibility [295] and a functional role in cartilage has been suggested despite its pseudogene status [296].

Paget's disease (PD) is a metabolic bone disease characterized by an imbalance between osteoclast and osteoblast activity that typically begins with excessive bone resorption followed by an increase in bone formation [297]. It has a predilection for the axial skeleton but any bone may be affected [298]. Several susceptibility genes for PD have been identified (Table 2-7). The gene most frequent linked to PD is *SQSTM1* which

encodes sequestosome 1 a multifunctional protein involved in the IL-1, TNF, and RANKL signaling pathways [297].

TNFRSF11A (TNF Receptor Superfamily Member 11a) and *TNFRSF11B* are genes which encodes the RANK protein and osteoprotegerin (OPG), respectively. Both RANK and OPG are involved in the RANKL/OPG/RANK signaling pathway, which is the principal regulator of osteoclastogenesis [299]. The *TNFRSF11A* gene is not only associated with PD, but also with familial expansile osteolysis (174810) and osteopetrosis (612301) and the *TNFRSF11B* gene could also be associated with bone mineral density and osteoporosis [300] and a gain of function mutation in this gene causes osteoarthritis with chondrocalcinosis [301]. The *ZNF687* gene was recently found in a whole exome sequencing study as associated with PD [302]. There is not much information about this gene, however it is known that it encodes a C2H2 (Cys2His2-like) zinc finger protein involved in the transcriptional regulator complex Z3 [303]. It has been suggested that probably this zinc finger protein is involved in bone cell proliferation and differentiation since it is up-regulated during osteoblast and osteoclast differentiation and is highly expressed during the regeneration of caudal fins in zebrafish [302]. Lastly, a linkage to the 5q31 region, designated by PD bone 4 (PDB4), seems to be associated with PD, however no candidate gene has been found in this region yet.

CHAPTER III: MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1 Collections- patients/families and associated data

The biobank Azores (AZORBIO) of the Specialized Service of Epidemiology and Molecular Biology (SEEBMO) has a collection of biological material (DNA; RNA; cartilage; plasma; serum; cell lines and urine) and associated data (clinical; epidemiological and genetic data) of Azorean patients with different pathologies. AZORBIO was founded in 1998 and has the facilities and equipment necessary for its operation. All the samples and data are manipulated in the same way according to standard operating procedures and all the samples are tested by a quality control process. All the collections have the documentation required by Portuguese law (Lei nº 12/2005), such as informed consent of all individuals [304]. The main objective of this biobank is to store biological products and associated data for further confirmation of the disease and for research.

3.1.1. Families DISH/CC

Eleven probands from ten families (AZ1-10) (Figure 2-1) were identified through a record review conducted at the Rheumatic Diseases Clinic of the Hospital of Santo Espírito da Ilha Terceira (HSEIT). The 11 probands and 71 family members (Figure 3-1 and table 3-1) were interviewed and examined for the presence of DISH and or CC by a rheumatologist (JBA). All participants gave informed consent, and standard X rays were taken from: knees, axial skeleton, wrists, hands, elbows, and pelvis. Pathological status was determined radiographically, with the diagnosis of DISH being made according to the Utsinger criteria [5], and the diagnosis of chondrocalcinosis on the classic radiological evidence of deposition of calcium in the fibrocartilage. All individuals were screened for secondary causes of chondrocalcinosis, using appropriate biochemical and genetic tests. The pedigrees and the associated data of the collaborating families were available in AZORBIO and were used in this work.

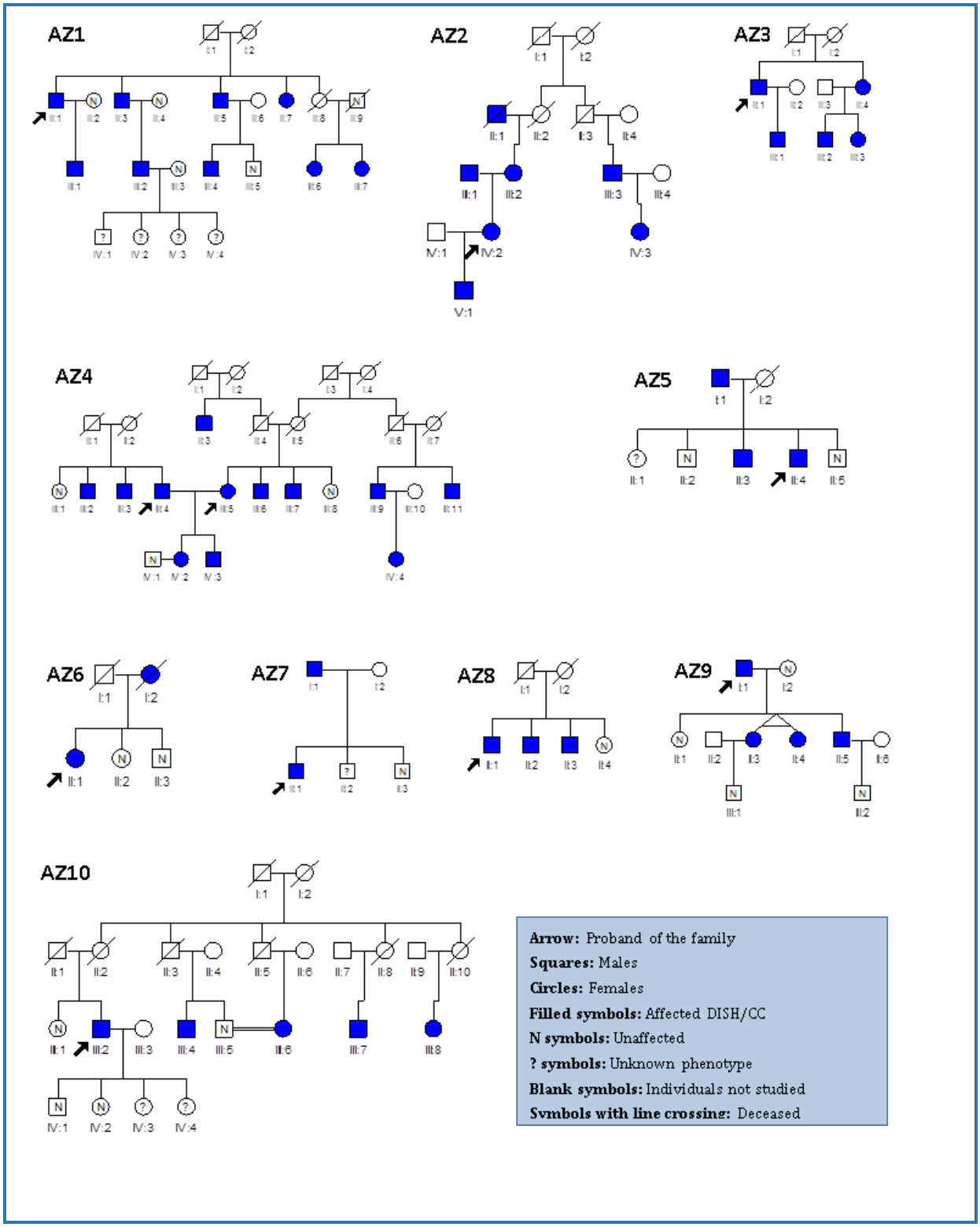


Figure 3-1. Azorean families included in the study.

Table 3-1. Azorean families included in the study and associated data (pedigree code; sex; age and other diseases). Probands are represented in bold.

Code	Sex	Age	DISH/CC	Other diseases	Code	Sex	Age	DISH/CC	Other diseases
AZ1					**AZ2**				
II:1	M	94	A		II:1	M	?	A	
II:2	F	90	UN		III:1	F	81	A	Lithiasis; DM
II:3	M	98	A		III:2	M	85	A	Lithiasis; polyarthritis
II:4	F	94	UN		III:3	M	94	A	
II:5	M	86	A	AS; DM	**IV:2**	F	58	A	Obesity
II:7	F	91	A	AS	IV:3	F	61	A	
II:9	M	?	UN	AS	V:1	M	33	A	
III:1	M	56	A		**AZ4**				
III:2	M	61	A		II:3	M	93	A	
III:3	F	57	UN		III:1	F	?	UN	
III:4	M	53	A	Obesity; AS	III:2	M	64	A	Lithiasis
III:5	M	56	UN		III:3	M	71	A	
III:6	F	71	A	AS	**III:4**	M	75	A	
III:7	F	76	A		**III:5**	F	70	A	DM; obesity; lithiasis
IV:1	M	31	?		III:6	M	68	A	Lithiasis
IV:2	F	30	?		III:7	M	81	A	
IV:3	F	34	?		III:8	F	77	UN	Kidney cramps
IV:4	F	36	?		III:9	M	90	A	
AZ3					III:11	M	87	A	
II:1	M	80	A	Sjogren	IV:1	M	53	UN	
II:4	F	89	A	Obesity	IV:2	F	48	A	
III:1	M	48	A	AS ;LBP	IV:3	M	43	A	
III:2	M	83	A		IV:4	F	58	A	DR
III:3	F	60	A	Periodontitis	**AZ5**				
AZ6					I:1	M	87	A	
I:2	F	?	A		II:1	F	62	?	
II:1	F	72	A		II:2	M	77	UN	Coxarthrosis
II:2	F	75	UN	LBP	II:3	M	58	A	
II:3	M	66	UN		**II:4**	M	55	A	
AZ7					II:5	M	52	UN	Crohn disease
I:1	M	93	A		**AZ8**				
II:1	M	61	A	AS	**II:1**	M	57	A	Lung cancer
II:2	M	67	?	LBP; AS	II:2	M	65	A	Parkinson
II:3	M	66	UN		II:3	M	62	A	AS
AZ10					II:4	F	62	UN	
III:1	F	68	UN		**AZ9**				
III:2	M	71	A	CC	**I:1**	M	86	A	Parkinson; Osteopoikilosis
III:4	M	60	A		I:2	F	82	UN	
III:5	M	76	UN		II:1	F	61	UN	
III:6	F	70	A		II:3	F	54	A	LBP
III:7	M	68	A		II:4	F	54	A	
III:8	F	77	A		II:5	M	58	A	
IV:1	M	46	UN		III:1	M	31	UN	
IV:2	F	45	UN		III:2	M	32	UN	
IV:3	F	42	?						
IV:4	F	35	?						

Abbreviations: M: male, F: female, AS: Ankylosing spondylitis. A: Affected; UN: Unaffected, CC: Chondrocalcinosis, DM: Diabetes mellitus, LBP: Low back pain, DR: Diabetic retinopathy, ?: Unknown.

3.1.2 DISH/CC patients not related

The 55 patients (36 males; 19 females) from this collection were identified through a record review conducted at the Rheumatic Diseases Clinic of HSEIT. Blood was obtained from all participants who gave informed consent. Standard X rays were taken from: knees, axial skeleton, wrists, hands, elbows and pelvis. All individuals were interviewed and examined for the presence of DISH and/or CC by a rheumatologist (JBA). Affected status was determined radiographically, with the diagnosis of DISH being made according to the Utsinger criteria [5], and the diagnosis of chondrocalcinosis on the classic radiological evidence of deposition of calcium in the fibrocartilage. The biological material and the associated data (Table 3-2) of these patients were available in AZORBIO.

Table 3-2. Patients with DISH/CC and associated data.

Code	Sex	Age	Other diseases	Code	Sex	Age	Other diseases
D1	M	72	Gastric cancer	**D29**	F	77	Mieloproliferative disease
D2	F	61		**D30**	M	60	Osteoid osteoma
D3	F	65		**D31**	M	86	
D4	F	70		**D32**	M	73	
D5	M	64	Parkinson disease; periodontitis	**D33**	F	66	
D6	M	66	Colorectal cancer	**D34**	F	74	
D7	M	54		**D35**	M	87	
D8	M	63		**D36**	F	70	Arterial disease
D9	M	61		**D37**	F	62	Osteoarthritis; DR
D10	M	59		**D38**	M	71	
D11	M	80	Sjogren syndrome	**D39**	M	70	DM
D12	M	88	DR	**D40**	F	81	
D13	M	79		**D41**	F	68	BC
D14	M	79		**D42**	F	56	
D15	M	65	Kidney cancer	**D43**	F	73	DR
D16	M	80	Prostate cancer	**D44**	F	64	BC
D17	M	62		**D45**	M	85	
D18	M	67	Lung cancer	**D46**	F	68	
D19	M	60		**D47**	M	65	Arterial hypertension
D20	M	67	Colorectal cancer	**D48**	F	78	
D21	M	87	Colorectal cancer	**D49**	F	72	BC
D22	M	70		**D50**	M	65	
D23	M	94		**D51**	M	64	
D24	M	69		**D52**	M	78	Colorectal cancer
D25	M	98		**D53**	F	91	Total hip replacement
D26	M	55		**D54**	M	86	Parkinson disease; osteopoikilosis
D27	M	66	Psoriatic arthritis	**D55**	F	77	DM;DR
D28	F	73					

Abbreviations: M: male, F: female, DM: Diabetes mellitus, DR: Diabetic retinopathy, BC: breast cancer.

3.1.3 Gitelman syndrome family

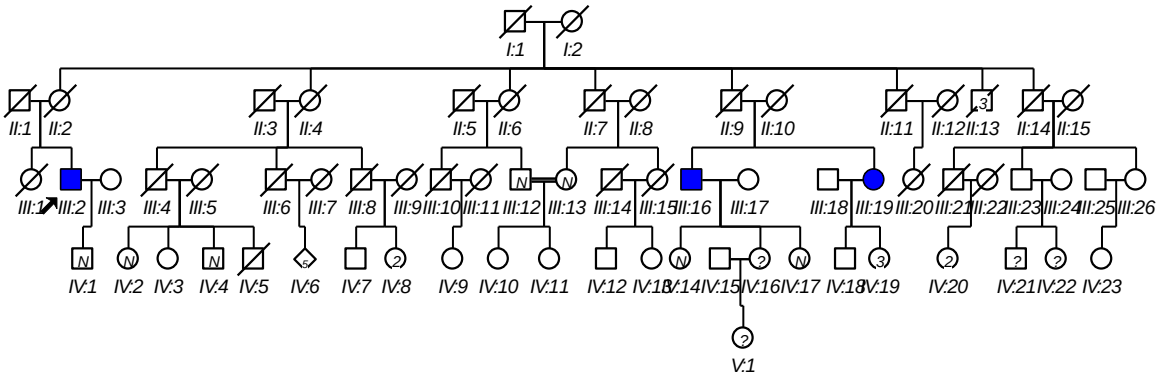


Figure 3-2. Gitelman family. The proband with GS and chondrocalcinosis is indicated by an arrow.

A proband with bilateral knee CC, hypomagnesemia and hypokalemia, was identified in the Rheumatic Diseases Clinic, HSEIT, Azores, Portugal. Blood was obtained from all family members who gave informed consent, and standard X rays were taken from: knees, axial skeleton, wrists, hands, elbows and pelvis. Proband and 13 family members (Figure 3-2) were interviewed and examined for the presence of CC by a rheumatologist (JBA). Pathological status was determined radiographically, with the diagnosis of CC on the classic radiological evidence of deposition of calcium in the fibrocartilage and Gitelman syndrome by the presence of a hypomagnesemia and hypokalemia. The biological material and the associated data (Table 3-3) of this family were available in AZORBIO.

Table 3-3. Gitelman syndrome family and associated data (code, sex, age, biochemical analysis and diseases). Reference values: Magnesium (1.5-2.5 mg/Dl); Potassium (3.3-5.1 mmol/L) and calcium (8.0-10.2 mg/dL). The proband are represented in bold.

Individuals			Biochemical analysis			Diseases	
Code	**Sex**	**Age**	**Magnesium**	**Potassium**	**Calcium**	**GS**	**CC**
III.2	**M**	**62**	**1,1**	**3,2**	**9,9**	**X**	**X**
III.12	M	75	2,1	4,7	9,1		
III.13	F	67	2,2	4,1	9,5		
III.16	M	75	2	4,6	9,5		X
III.19	F	79	1,9	4,7	9,7		X
IV.1	M	35	2,3	3,7	?		
IV.2	F	54	2,2	4,2	9,7		
IV.4	M	46	1,9	4	9,2		
IV.14	F	51	1,9	4	9		
IV.16	F	49	2,2	4,1	9,1		
IV.17	F	45	1,9	4,3	9		
IV.21	M	37	2,1	4,1	10		
IV.22	F	36	2	3,7	9,7		
V.1	F	25	2	4,7	9,5		

Abbreviations: M-Male, F- Female, GS- Gitelman Syndrome; CC-Chondrocalcinosis.

3.1.4 Ankylosing Spondylitis (AS) patients

The 25 ankylosing spondylitis patients in this collection were identified through a record review conducted at the Rheumatic Diseases Clinic of HSEIT. Blood was obtained from all participants who gave informed consent and axial skeletal radiographs and CT scans of the sacroiliac joints, as needed, were taken. The HLA–B27 typing was also performed in all patients. All individuals were interviewed and examined for the presence of AS by a rheumatologist (JBA). Affected status was determined radiographically, with the definitive diagnosis of AS being made in accordance with the modified New York criteria [305].

The biological material and the associated data (Table 3-4) of these patients were available in AZORBIO:

Table 3-4. AS population and associated data (sex; age and other diseases).

Code	Sex	Age	Other diseases	Code	Sex	Age	Other diseases
AS1	F	57	LBP	**AS14**	M	51	DISH/CC; colorectal cancer
AS2	M	58		**AS15**	M	58	
AS3	F	47	Polyarthritis; total hip replacement	**AS16**	M	73	Dementia
AS4	M	40		**AS17**	M	70	Psoriatic arthritis; uveitis
AS5	M	46		**AS18**	M	91	
AS6	F	55	Uveitis	**AS19**	F	74	Uveitis
AS7	M	71		**AS20**	M	37	
AS8	M	63		**AS21**	M	47	
AS9	M	58		**AS22**	F	65	Uveitis; vasculitis
AS10	F	81	DR	**AS23**	F	37	
AS11	M	82	Psoriatic arthritis	**AS24**	M	65	
AS12	M	57		**AS25**	M	90	
AS13	M	60	Psoriatic arthritis				

Abbreviations: M- male, F- female, LBP- Low back pain, DR- Diabetic retinopathy, CC- chondrocalcinosis, DISH- diffuse idiopathic skeletal hyperostosis.

3.1.5 Control population without DISH/CC

This population cohort consists of 36 individuals (16 Males and 20 Females), older than 65 years of age, living in the Terceira Island, that were being followed by the Rheumatic Diseases Clinic and Oncology Service of HSEIT. Blood was obtained from all participants who gave informed consent, and radiographs of the axial spine, pelvis, knees, wrists, hands and elbows (assessed by a rheumatologist (JBA) to confirm the absence of calcifications and/or ossifications). The biological material and the associated data (Table 3-5) of these patients were available in AZORBIO.

Table 3-5. Population control without DISH and or CC and associated data (sex; age and other diseases).

Code	Sex	Age	Diseases	Code	Sex	Age	Diseases
C1	M	64	RA; hypothyroidism	**C19**	M	102	
C2	F	69	Chronic lymphoid leukemia	**C20**	F	57	
C3	M	76	Colorectal and lung cancer	**C21**	F	74	BC
C4	F	68	BC	**C22**	F	72	Myelofibrosis; myeloproleferative disease
C5	F	77	Non hodgkin lymphoma	**C23**	F	72	
C6	F	61		**C24**	F	63	
C7	M	67	Colorectal cancer	**C25**	F	72	BC
C8	F	75	BC	**C26**	F	66	Colorectal cancer

Code	Sex	Age	Diseases	Code	Sex	Age	Diseases
C9	F	59	Colorectal cancer	**C27**	M	69	Gastric cancer
C10	M	73		**C28**	F	68	Lymphoma
C11	M	74		**C29**	F	59	BC
C12	M	63	Lung cancer	**C30**	F	63	BC
C13	M	62	Myelodysplastic syndrome	**C31**	F	70	BC
C14	M	80	Lung and colorectal cancer	**C32**	F	68	BC
C15	M	60	Lymphoma	**C33**	F	67	
C16	M	63	Pancreas cancer	**C34**	F	60	Colorectal cancer
C17	M	82	Non hodgkin lymphoma	**C35**	M	69	Non hodgkin lymphoma
C18	M	63	BC	**C36**	M	64	Lung cancer

Abbreviations: M- male, F- female, RA- Rheumatoid arthristis, BC- breast cancer.

3.1.6 Representative population of Terceira Island

Presently, Terceira island is an autonomous Portuguese region with approximately 60,000 inhabitants. The two collections used in this thesis consist of individuals living on Terceira Island.

3.1.6.1 Randomized cohort

This population cohort consists of 124 individuals (46 males; 78 females; mean current age 66; range, 35-100), older than 18 years of age, living in the Terceira Island. The randomization was performed by "Serviço Regional de Estatística dos Açores" using the census carried out in 1981. All participants were interviewed about: demographic information and personal and familial diseases. Blood was obtained from all participants who gave informed consent and individuals who reported back pain (11 males; 19 females), radiographs of the axial spine was also performed. The biological material and the associated data (Table 3-6) of these patients were available in AZORBIO.

Table 3-6. Individuals from the randomized population and associated data (sex, age and diseases).

Code	Sex	Age	Diseases	Code	Sex	Age	Diseases
R1	F	60	LBP; phlebitis	**R63**	F	88	LBP
R2	F	53	Hyperparathyroidism	**R64**	F	74	DM
R3	M	61		**R65**	F	76	RA; hypercholesterolaemia
R4	M	46	Knee pain	**R66**	F	88	DM
R5	M	64		**R67**	F	54	LBP
R6	M	85	Colorectal cancer	**R68**	F	82	LBP
R7	M	49		**R69**	F	96	Cardiac disease
R8	M	63	Colorectal cancer	**R70**	F	96	LBP
R9	M	85	LBP	**R71**	F	61	Epilepsy
R10	M	53	Psoriasis; AS	**R72**	F	61	LBP
R11	F	100	LBP; poliomyelitis	**R73**	F	57	
R12	F	90	Juvenil arthritis	**R74**	F	70	Hand osteoarthritis
R13	F	58		**R75**	F	94	
R14	F	46	LBP	**R76**	F	77	
R15	F	60		**R77**	F	67	
R16	F	76		**R78**	F	78	LBP
R17	M	61	LBP; kidney cramps; psoriasis	**R79**	F	54	
R18	M	56		**R80**	F	90	LBP; colitis
R19	F	70		**R81**	F	54	BC
R20	M	76	LBP	**R82**	F	63	Poliomyelitis
R21	F	53		**R83**	F	68	Left sacroiliitis; LBP; right sciatica
R22	F	52		**R84**	F	73	LBP; hypercholesterolaemia; DM; BC
R23	F	89	DM; LBP	**R85**	F	48	Hypertension; transient schemic attack
R24	F	56	Deformities in tibias	**R86**	F	47	
R25	F	60	LBP; AS	**R87**	F	35	
R26	M	78	DM	**R88**	F	65	LBP
R27	M	87		**R89**	F	64	Kidney cramps; BC
R28	M	84		**R90**	F	71	
R29	M	55	LBP; leucopeny	**R91**	F	53	AS
R30	M	65		**R92**	F	61	
R31	F	53	LBP; multi brain strokes	**R93**	F	86	
R32	F	56	Juvenil stroke	**R94**	F	68	Anemia
R33	M	89		**R95**	F	74	
R34	M	49	LBP	**R96**	F	65	
R35	M	73	Cardiac disease; DM; hand deformities; lung cancer	**R97**	F	70	Enteritis; LBP
R36	M	96	Chronic obstructive pulmonary disease	**R98**	F	61	
R37	M	71	LBP; CC	**R99**	F	83	LBP; coxofemoral fracture
R38	M	69	LBP	**R100**	F	93	
R39	M	66		**R101**	F	81	AS
R40	M	67		**R102**	F	79	DM; osteoarthritis; RA; LBP
R41	F	93		**R103**	F	62	
R42	M	67	Kidney pain	**R104**	F	76	
R43	F	80		**R105**	M	42	
R44	F	55	Colitis	**R106**	F	46	AS
R45	F	63		**R107**	F	81	BC
R46	F	64		**R108**	F	45	Psoriasis

Code	Sex	Age	Diseases	Code	Sex	Age	Diseases
R47	F	55		**R109**	M	46	
R48	M	63	LBP	**R110**	M	75	
R49	M	42		**R111**	M	51	
R50	M	81		**R112**	M	49	
R51	F	54		**R113**	M	42	
R52	F	71		**R114**	F	57	
R53	F	45	Scoliosis	**R115**	F	50	Polyarthritis
R54	M	78		**R116**	F	74	
R55	M	72	LBP; AS	**R117**	F	41	Knee swelling; LBP
R56	M	44	LBP	**R118**	M	89	
R57	M	84	LBP; osteoporosis	**R119**	M	55	Ischemic stroke
R58	M	67		**R120**	F	79	Psychiatric disorder
R59	M	77	Diabetes; two myocardial	**R121**	F	47	
R60	M	68		**R122**	M	42	
R61	M	92	Cardic disease; LBP	**R123**	M	42	
R62	F	51		**R124**	F	77	

Abbreviations: M- male, F- female, AS- ankylosing spondylitis; CC- chondrocalcinosis; LBP- Low back pain; BC- breast cancer; RA- Rheumatoid arthritis, DM- Diabetes mellitus.

3.1.6.2 Two regions – Angra do Heroísmo and Praia da Vitória

This population cohort consists of 375 individuals (85 males; 290 females; mean current age 55; range, 25-91), older than 18 years of age, living in the Terceira Island, that were attended in clinical laboratories located in the two main municipalities of the Terceira island; Angra do Heroísmo and Praia da Vitória. All participants who gave informed consent were interviewed about breast cancer and other inherited diseases, socio and demographic information. Blood was obtained from all participants who gave informed consent. The biological material and the associated data (Table 3-7) of these patients were available in AZORBIO.

Table 3-7. Representative population of two regions of Terceira Island and associated data (sex, age and diseases).

Code	Sex	Age	Diseases	Code	Sex	Age	Diseases	Code	Sex	Age	Diseases
2R1	F	56		**2R126**	M	57		**2R251**	F	62	
2R2	F	41		**2R127**	M	37		**2R252**	F	75	
2R3	F	48		**2R128**	M	68		**2R253**	F	58	
2R4	F	47		**2R129**	M	84		**2R254**	F	56	Bilateral uveitis
2R5	F	47		**2R130**	M	56		**2R255**	F	74	
2R6	F	47		**2R131**	M	66		**2R256**	F	43	
2R7	M	56		**2R132**	M	46		**2R257**	M	34	
2R8	F	41		**2R133**	F	27		**2R258**	F	72	
2R9	F	35		**2R134**	M	45		**2R259**	F	72	
2R10	M	79		**2R135**	M	59		**2R260**	F	73	
2R11	F	84		**2R136**	M	59		**2R261**	F	59	

Code	Sex	Age	Diseases	Code	Sex	Age	Diseases	Code	Sex	Age	Diseases
2R12	M	63		**2R137**	M	86		**2R262**	F	80	
2R13	M	65		**2R138**	M	64		**2R263**	F	70	
2R14	M	78		**2R139**	F	78	RA	**2R264**	F	46	
2R15	M	51		**2R140**	M	72	Chronic lymphoid leukemia	**2R265**	F	53	
2R16	F	75		**2R141**	M	59		**2R266**	F	51	
2R17	M	73		**2R142**	M	82		**2R267**	F	70	
2R18	F	52		**2R143**	M	36		**2R268**	F	71	
2R19	M	78		**2R144**	F	87		**2R269**	F	77	
2R20	F	56		**2R145**	M	73		**2R270**	F	49	
2R21	F	60		**2R146**	M	86	Dementia	**2R271**	F	65	
2R22	F	61		**2R147**	F	46		**2R272**	F	69	
2R23	F	43		**2R148**	F	62		**2R273**	F	56	
2R24	F	55		**2R149**	F	43		**2R274**	F	32	
2R25	F	65	Polyarthritis	**2R150**	F	45		**2R275**	F	86	
2R26	F	52		**2R151**	M	57		**2R276**	F	61	RA
2R27	F	46		**2R152**	F	69		**2R277**	F	61	Lacunar stroke
2R28	F	60	DM	**2R153**	F	45		**2R278**	F	59	
2R29	F	59	AS; uveitis	**2R154**	M	68		**2R279**	F	70	
2R30	F	45		**2R155**	F	47		**2R280**	F	66	
2R31	F	66		**2R156**	F	56		**2R281**	F	49	
2R32	F	47	Dysuria	**2R157**	F	52		**2R282**	F	58	
2R33	F	74		**2R158**	F	58		**2R283**	M	40	
2R34	F	50		**2R159**	F	56		**2R284**	F	67	
2R35	F	52		**2R160**	F	63		**2R285**	F	55	
2R36	F	62		**2R161**	M	35		**2R286**	M	55	
2R37	F	46		**2R162**	F	50		**2R287**	M	47	Psoriatic arthrtis; gonarthrosis
2R38	F	45		**2R163**	F	53		**2R288**	F	69	
2R39	F	55		**2R164**	F	26		**2R289**	F	70	
2R40	F	59	AS	**2R165**	F	66		**2R290**	F	60	
2R41	F	37		**2R166**	F	53		**2R291**	M	64	
2R42	F	44		**2R167**	M	60		**2R292**	F	45	
2R43	F	36		**2R168**	F	55		**2R293**	F	65	
2R44	F	45		**2R169**	F	59		**2R294**	F	67	
2R45	F	41		**2R170**	F	62	AS; RA	**2R295**	F	34	
2R46	M	75		**2R171**	F	52		**2R296**	F	81	
2R47	F	32		**2R172**	F	47		**2R297**	F	79	
2R48	F	52		**2R173**	F	53		**2R298**	F	72	
2R49	F	39		**2R174**	F	56		**2R299**	F	65	
2R50	F	42		**2R175**	F	57		**2R300**	F	81	
2R51	M	56		**2R176**	F	72		**2R301**	F	57	BC
2R52	F	34		**2R177**	M	37	Bilateral uveitis	**2R302**	F	51	
2R53	M	34		**2R178**	F	52		**2R303**	F	27	
2R54	F	76		**2R179**	F	44		**2R304**	M	38	
2R55	F	43		**2R180**	F	86		**2R305**	F	62	

Code	Sex	Age	Diseases	Code	Sex	Age	Diseases	Code	Sex	Age	Diseases
2R56	F	41		**2R181**	F	57		**2R306**	F	39	
2R57	F	61		**2R182**	F	77		**2R307**	F	41	
2R58	F	38	polyarthralgia; acrocyanosis; ischemic feet	**2R183**	M	38		**2R308**	F	57	
2R59	F	56		**2R184**	F	76	Lacunar stroke	**2R309**	F	53	
2R60	F	38		**2R185**	F	58		**2R310**	F	34	
2R61	F	39		**2R186**	F	78		**2R311**	F	25	
2R62	M	55		**2R187**	F	87		**2R312**	M	50	
2R63	F	46		**2R188**	F	66		**2R313**	F	62	
2R64	M	35		**2R189**	F	76	DISH/CC; colorectal	**2R314**	F	53	
2R65	F	39		**2R190**	F	57		**2R315**	M	69	
2R66	F	35		**2R191**	F	79		**2R316**	M	47	
2R67	M	56		**2R192**	F	44		**2R317**	M	45	
2R68	M	46		**2R193**	F	65		**2R318**	M	41	
2R69	F	43		**2R194**	F	48		**2R319**	F	25	
2R70	F	40		**2R195**	F	77		**2R320**	F	45	
2R71	M	32		**2R196**	F	70		**2R321**	F	48	
2R72	F	59		**2R197**	F	52	Oligoarthritis	**2R322**	F	44	
2R73	F	36		**2R198**	F	67		**2R323**	F	61	
2R74	M	75	Ischaemic stroke	**2R199**	M	76		**2R324**	F	39	
2R75	F	44		**2R200**	F	58		**2R325**	M	41	
2R76	F	58	Convulsion	**2R201**	F	48		**2R326**	M	57	
2R77	F	50		**2R202**	F	52		**2R327**	M	31	
2R78	M	42		**2R203**	F	62		**2R328**	M	40	
2R79	M	67		**2R204**	F	65		**2R329**	F	38	
2R80	F	45		**2R205**	F	76	DR	**2R330**	M	44	
2R81	F	39		**2R206**	F	54		**2R331**	M	42	
2R82	M	81		**2R207**	M	64		**2R332**	M	31	
2R83	F	55	AS	**2R208**	F	72		**2R333**	M	64	
2R84	F	54		**2R209**	F	69	Peripheral arterial disease	**2R334**	F	62	
2R85	F	54		**2R210**	F	91		**2R335**	F	31	
2R86	F	34		**2R211**	F	48		**2R336**	F	83	
2R87	F	26		**2R212**	F	39		**2R337**	F	54	Colorectal
2R88	M	84	AS	**2R213**	F	54		**2R338**	M	69	Neck pain
2R89	F	47		**2R214**	F	29		**2R339**	F	55	
2R90	F	41		**2R215**	F	63	DISH/CC	**2R340**	F	36	
2R91	M	78		**2R216**	F	52		**2R341**	F	35	
2R92	M	65		**2R217**	F	68		**2R342**	F	62	
2R93	M	49		**2R218**	F	41		**2R343**	F	49	Cerebral venous thrombosis
2R94	F	55		**2R219**	F	29		**2R344**	M	43	
2R95	F	85		**2R220**	F	68		**2R345**	F	37	
2R96	M	73		**2R221**	F	71		**2R346**	F	32	Leukorrhoea

Code	Sex	Age	Diseases	Code	Sex	Age	Diseases	Code	Sex	Age	Diseases
2R97	F	59	Uveitis	**2R222**	F	54	Cerebellar infarction left	**2R347**	F	55	RA
2R98	F	52	BC	**2R223**	F	73		**2R348**	F	44	
2R99	F	53		**2R224**	F	55		**2R349**	F	38	DISH/CC
2R100	F	54		**2R225**	F	81		**2R350**	F	37	
2R101	F	59		**2R226**	F	55		**2R351**	F	35	
2R102	M	38		**2R227**	F	61		**2R352**	F	40	
2R103	F	66		**2R228**	M	81		**2R353**	F	33	
2R104	F	63		**2R229**	F	64		**2R354**	F	25	
2R105	F	56	AS	**2R230**	F	70	Betathalassemia syndrome	**2R355**	F	32	
2R106	F	48		**2R231**	F	74		**2R356**	F	79	
2R107	F	51		**2R232**	F	53		**2R357**	F	42	
2R108	M	35		**2R233**	F	57		**2R358**	F	44	
2R109	M	53		**2R234**	F	59		**2R359**	F	25	
2R110	M	51		**2R235**	F	64		**2R360**	M	29	
2R111	F	49	Multi brain strokes	**2R236**	F	58		**2R361**	M	68	
2R112	F	55		**2R237**	F	55		**2R362**	F	88	
2R113	F	64		**2R238**	F	54		**2R363**	F	56	
2R114	F	72		**2R239**	F	53		**2R364**	F	35	
2R115	F	48		**2R240**	F	70		**2R365**	M	34	
2R116	F	59		**2R241**	F	90		**2R366**	M	75	
2R117	F	64		**2R242**	F	62		**2R367**	F	37	Cerebral venous
2R118	F	57		**2R243**	F	61	DISH/CC	**2R368**	F	27	
2R119	F	60		**2R244**	F	49		**2R369**	F	47	
2R120	F	27		**2R245**	F	61		**2R370**	F	41	
2R121	F	61		**2R246**	F	67	BC	**2R371**	F	63	
2R122	M	64		**2R247**	F	34		**2R372**	M	39	
2R123	M	49		**2R248**	F	91		**2R373**	F	45	
2R124	F	78		**2R249**	F	63	Spherocytosis	**2R374**	F	51	
2R125	M	66		**2R250**	F	83	RA	**2R375**	F	61	

Abbreviations: M- male, F- female, AS- Ankylosing spondylitis; RA- Rheumatoid arthritis; DR- Diabetic retinopathy; BC- Breast cancer, CC- chondrocalcinosis, DISH- diffuse idiopathic skeletal hyperostosis.

3.1.7 Total hip replacement group

This collection of 53 individuals (34 male, 17 female; mean current age, 71 years; range, 47-93 years) (Table 3-8) was obtained from patients undergoing total hip replacement surgery. Informed consent was obtained from these patients for use of their rejected tissue in research. Sterile cartilage sections (of approximately 3 mm diameter) from the coxofemoral articular cartilage were prepared with a scalpel and a cutter and immediately stored at -80ºC in RNA later.

Table 3-8. Total hip replacement group.

Code	Sex	Age	Diseases	Code	Sex	Age	Diseases
THR1	M	81	DR	**THR28**	M	65	
THR2	F	79		**THR29**	M	47	
THR3	M	66		**THR30**	F	78	
THR4	F	67	DISH, BC	**THR31**	M	53	
THR5	M	57		**THR32**	M	73	
THR6	M	74		**THR33**	F	82	
THR7	M	56		**THR34**	F	65	
THR8	M			**THR35**	M	82	CC (knees)
THR9	M	72		**THR36**	F	64	CC (knees)
THR10	F	91		**THR37**	F	72	
THR11	M	59		**THR38**	F	71	
THR12	F	84		**THR39**			
THR13	M	79	DISH	**THR40**	F	68	
THR14	M	76		**THR41**	F	59	
THR15	M	79		**THR42**	F	89	
THR16	M	70		**THR43**	M	77	Osteoarthritis
THR17	M	77		**THR44**	F	53	
THR18	M	72		**THR45**	F	59	
THR19	M	73		**THR46**	F	56	
THR20	M	77	Hypertension	**THR47**	M	71	Lithiasis
THR21	M	74		**THR48**		50	
THR22	M	87	Prostate cancer	**THR49**	M	84	
THR23	M	81		**THR50**	M	72	
THR24	M	83		**THR51**	M	69	RA
THR25	M	69		**THR52**	M	79	
THR26	M	62		**THR53**	F	93	DISH/CC; AS
THR27	M	70					

Abbreviations: M-male, F- female, AS- Ankylosing spondylitis; R- Rheumatoid arthritis, D- Diabetic retinopathy; BC- Breast cancer, DISH- diffuse idiopathic skeletal hyperostosis.

3.2 Methods

3.2.1 Gene sequencing

3.2.1.1 DNA extraction and quantification

Genomic DNA was obtained from peripheral blood cells in EDTA (Ethylenediamine tetraacetic acid) anticoagulant, using a standard Salting-Out extraction method [306], and an automatic procedure (EZ1;Qiagen) according to the manufacturer's instructions. The DNA concentration and A260:A280 ratio was determined using the NanoVue spectrophotometer (GE Healthcare).

3.2.1.2 Amplification of regions of interest

PCR oligonucleotide primers were designed using the software Primer3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) for intronic sequences with the objective of amplify/sequence fragments from the following genes:

SLC12A3, *RSPO4*, *ENPP1*, *PPP2R2D*, *PLCG2*, *AMER3*, *FLNC*, *FGF2*, *COL11A2*, *VDR*, *BMP4* and *BMP2*. The primers used to amplify the *LEMD3* gene (exon 13) were previously described by Hellemans and collaborators [307] and primers for *ABCC6* gene fragments described by Miksch et al [308] and Cai et al [309]. PCR reactions were optimized for all primer sets (Table 3-9) in order to obtain the expected single reaction product.

Table 3-9. Forward and reverse oligonucleotide primers (5'→3') used in PCR reactions and sequencing of *SLC12A3, RSPO4, LEMD3, ENPP1, PPP2R2D, PLCG2, AMER3, FLNC, FGF2, COL11A2, VDR, BMP4, BMP2* and *ABCC6* genes.

Gene	Exon	Forward	Reverse
SLC12A3	1	CTCAGAAGAGCCACTCCAGG	GAGGTCACACAGCAGGGAAG
	2	AGTGGGCTGGATGCAGAGA	AGAGCTGAGCCTGGATGGA
	3	AGTGGGTGAAGAAGGGACC	AGGATTCAGGCAAGCTGG
	4	AGATGAACGTAGGTCGCATGG	CAGGATTAGGAGCCCACGAG
	5	CACGAGATGGCCTCAGCTATC	TGGTTCCTGATGGGTGAAGTC
	6	ATCGTCCTAGCAGAGTGCACC	CACGTGACCACCTCCATGTC
	7	GTGAATAATGGAGAAACGGGC	TTCCTGGGTAGAGAGTCCCTG
	8	ACTGGGAGGATGGGATTACC	CAGGATTCTGCTCATAGCCC
	9	GCCTAATGTCCTCCGTCCCT	TGCTCTGATCATTGCCAAGATAC
	10	ATCATCTGCAGCACCTCGC	CAGTGTCCACCCACAAGTCG
	11	CGCAGTAGGGAATGAAGTGC	CCTATTGTGCCTCTAGCCCAG
	12	AAACAGACACCAGGACCCAG	TGCCCACTAACTGTCAGGC
	13	CAGTCCTTGGCAGAGTTGC	CTGACCTCAAGTGATCCGC
	14	CCTAGAAAGAGGCTCGACTGC	GTTTCTTGCCACATTGGGAG
	15	AGAAGGCCGACATTACCTCTG	GTCTAGGCTTGGAAACTCCCA
	16	CATGTAGGGTCATGCTGGTG	GGCTGGTCTCAAACTCCTGA
	17	GTGAAGGCAGCTGGTGATGT	CAAGCCGTAAGTCCTGTAGGG
	18	TTGAGAATCAGCACATCTGGAG	CAATGGGCCCAAATTAACAG
	19	TGGTAGGAAGCAGAGCCAACT	AACTTTCTGGGAGTGGGTGG
	20	CCTGTCAAGGAGGAACCCA	AGTGCCCTGAGCTCTGAGTG
	21	TTCCTGTTCCACCTGCCA	GGCGACTTCAGCTCTTCTCTC
	22	CACATAGTGCTCTGTCCTGAGTG	TTTGGGACAATCTCAGTGCC
	23	GCAGAGGTTGCAGTGAATCG	GTCTCCAGGCACACAGTTGG
	24	CCTCAACCCACTTTCTCGTC	AGCTCAAGACATGCAGGACA
	25	AATGAGGCCATAGACGTGGT	AGCTGAGACACCTGACTCTGG
	26	CTGAGGGACGGTAAACAGAC	AGAGGCAATCAAGTTCCAAC
RSPO4	1	CAACGCCCTCACTAGACCTG	AGCCTCAATCTCCCCATCTTA
	2	TGACCATCTCTCTCTCCCTTTC	GAGCCCAGCACTAGCATAGAAC
	3	GGGGTGTCCCTGGCTCTA	GCCCCTAACATCTCTCACCA
	4	TCTGTCTGTCTCTCCTTTCACC	CAGCCTCGTGTGCCTGTC
	5	GCACCCTTGTCTTTCAGGACT	AAGAGTAAGAGGAGAGGAGGAGAA
LEMD3	13	CTTTTACCACAGTTAATTTTCTGC	GAACCTTAAGACTTCTGGAACG
ENPP1	4	CATGGTAGTGGCAGATTCTG	TTCAGCTAATATAGTTGGCC
PPP2R2D	7	GCCTGAAGATCCCAGCAGTA	CCTATGAGCGTCCTCCTCTG

Gene	Exon	Forward	Reverse
PLCG2	19	CCCACTGACAGCCTGGAG	CTGAAGTTCCCCCTTGTCTCT
	21	TGAGGTTTCAACTCCTCTATCAAA	AACAGAGTCAGGGTGGGAAT
	33	CCACTGCTGATGGTGAAATC	TCTTACATTTCCAGCTCATCCTC
AMER3	3	GAGGGCTACTATGATTCCTTCTCG	AAGCTGCATATGGACAGTGG
FLNC	26	TGTCATCGGCTTCTGGGAAC	ATCCCCATTGTCCCGGATGT
FGF2	3´UTR	GCTTTAGGCGGCAGATGATA	ACACAGCGGTTCGAGAAGTT
COL11A2	6	ACCTTCCAATTACCCCATCC	TGGGAGAAAGGGAAGAAATG
VDR	5	CTGGCACTGACTCTGGCTCT	TGCAGCCTTCACAGGTCATA
BMP4	7	GGGACCAGTGAAAACTCTGC	GGGGGCTTCATAACCTCATAA
BMP2	3	TTCCGAGAACAGATGCAAGA	TCAAAACTTTCCCACCTGCT
ABCC6	5'UTR-LR	TCCTGGAAATTGCTGGGTCCAAAGTGT TTAGGAAGTCT	CAGCTCACCTGCCCAGGGGGCCAG GCAACT
	1	CCGAGCAGTCTGCCCAGAGACTT	GGGGGTCTCTCCTCTCCCCAGTAT
	2	TGGCCCCCTGGGCAGGTGAG	GTCCCCTGCCTCCCCCGAACA
	3-4-LR	CCGGGGCTACCTCCGGATGTC	GGCCTGTAAGACAGGAAATTGTGTTG ATAAGA
	3	CCGCCTACCAGTTTGCTGTGAC	GGAGCCTCTTCTCTTCCCCTTGT
	4	CTGCTGCTTTGCCTGCCACAGT	GTGCGGGAGTGGATTTTGTGTCTCT
	5	GTCCCCAGAGTGGGCACTGAC	CTTTTGGTCACCTGGGGGAGAC
	6	TCCTGTCTTCCTACCCTTGCCACAT	GGCCATGGCTGGGAATCAGAG
	7	GCCAGGATCCTGCAGGGGTGAA	CGCACCCGGCCAATGATGAG
	8-9 LR	AGGCTAGATCCACACCCACCCATCTCCACTGT	CGTCCACGGACACCAGATTGACCACATCAC
	8	CCGCTGGCGGCTGAGAGTAT	GGCCCTGGAAGGATGCCACTA
	9	TTGGGCAAAGGCAACACCCTTAG	ACTGCTTTTCCTGGCTGGGAAGAC
	10-LR	GAGCCCTGAGAGGTTGGCCTAAGAGA CTTTACTC	TTCCCCCTAAAATGTTCTCCTCTTGTGTT TTGTG
	10	CCACCTGGGGCATCCCTCTG	GGGGAAGGACGAGGGGGAGAA
	11	TGCTCTGGTTCACGTGCCTCTG	TCAGCTCTCCCCTCCCCATCTC
	12	GGTGAGATGAATGGGATTTGCTGAAG	GGGGGGCTCCACCTACCTCAC
	13	GCTTGCCCAGGCTGCCCTATC	GGTAGGGAAGCTGGAGCCAGGTGTA
	14	GCCACACATCTTGAGACACCGACAC	CCAGTACTGATGCTGGCTTGCCATTA
	15	ATGGTGCCTGGGGGCCTCTC	GCAGGAGCCCCATGCATCTTCT
	16	TCAGCTCCGTCTGGGGCTCATC	GTGGGAAGGCAGCGAGGAAGTG
	17	CAGCTCCCACTGCTCCTCAAAAC	TCCATCATACTGCCCATGATGAGTC
	18	AGCCTGGGCACCCCAGTTTC	AAACTTGGGTTAGGACTGGATGCTAAGT
	19	CCACATGCTTTGGCTTCCCAAAGTGT	AGGGTGTGGCCAGAGCACTCCATTC
	20	AAGGCCACATAGTCAGTGGGTGTCA	GCGGGTGGTCCCTTCAGCTACT
	21	TGGCTGTCAGTGGGCCTGAG	GGTGAGTATCACTGCCAAGTGCTACA
	22	TCCCATCTGCCATGGGCATGTTTT	TTTGCACACTGTTCCAGGGGGACAG
	23	CACCATGGGGTAGCGGGAGAGAC	GGGAATTCTAGGAACAGCCCCTAGATGTC
	24	GGCTCTCTGTGCTTCTGGAAACTA	GGATATGGATGAATTGCAAGGTCTT
	25	TCCTTGTGCCCAGAGAAGCATCTC	CCACTAGCAGGGGTCCGACAGTC
	26	GGCTGTTGCAAGCCCTCAAGTG	AAACTCCAAAGCCTGTAGCAGATGTCA
	27	GAAGCTGATAGAGGTGGGCCATCTTG	GGTTTAGGGCCTTGTCCCTGGAGTC
	28	GAGGGATGGATAGACAGATCTCGGGTACA	ATCCGCAGAGAGCCAGGGAACAG
	29	GGTGGAGGGGGTGGGCAAAGA	GGCATGGCCATCCCCTCCTCTC
	30	CTGTTTCTGGGCACACCCACACATC	CCAGGACTGCCTCCGCCTCCT
	31	CGCAGACACACTGGGCTCTCACA	GATGACCACGGGTCACTTCCATCTC

Abbreviations: LR- Long range.

PCR amplification for each gene fragment were performed using the PCR System 9700 (Applied Biosystems) using the conditions described in table 3-10. Long range PCR reaction for E08-E09-LR in *ABCC6* gene were performed using Long and Accurate (LA-Taq) polymerase (TAKARA) and a PCR cycle protocol according to the manufacturer's instructions. PCR products were subjected to electrophoreses on a 1.7% agarose gel (Seakem), at 125V for 25 minutes and stained with GelRed (Olerup) to verify the presence of specific PCR products of the expected size (Table 3-10).

Table 3-10. PCR conditions, annealing temperature and PCR product size for *SLC12A3, RSPO4, LEMD3, ENPP1, PPP2R2D, PLCG2, AMER3, FLNC, FGF2, COL11A2, VDR, BMP4, BMP2* and *ABCC6* genes. To amplify the exon 18 of *SLC12A3* and exon 26 of *ABCC6*, 2 and 2.5 µl of DMSO were added.

Gene	Exon	PCR conditions							At (ºC)	bp
		Buffer	MgCl2 (mM)	dNTPs (mM)	Primers (F+R) pmol	BSA (mg/ml)	GoTaq (U)	DNA (ng)		
SLC12A3	1	1X	2	0.15	0.2	0.3	0.04	50	65-55*	579
	2	1X	2	0.15	0.2	0.3	0.04	50	65-55*	292
	3	1X	2	0.15	0.2	0.3	0.04	50	65-55*	279
	4	1X	2	0.15	0.2	0.3	0.04	50	65-55*	240
	5	1X	2	0.15	0.16	0.3	0.02	50	55º	388
	6	1X	2	0.15	0.2	0.3	0.04	50	65-55*	263
	7	1X	1	0.15	0.2	0.3	0.04	50	50º	275
	8	1X	2	0.15	0.2	0.3	0.04	50	65-55*	309
	9	1X	1	0.2	0.8	-	0.01	30	56º	358
	10	1X	2	0.15	0.32	0.3	0.02	50	55º	429
	11	1X	2	0.15	0.32	0.3	0.02	50	55º	254
	12	1X	2	0.15	0.2	0.3	0.04	50	65-55*	378
	13	1X	0,8	0.16	0.64	-	0.01	50	58º	395
	14	1X	1	0.15	0.2	0.3	0.04	50	50º	370
	15	1X	2	0.15	0.2	0.3	0.04	50	65-55*	272
	16	1X	2	0.15	0.2	0.3	0.04	50	65-55*	476
	17	1X	2	0.15	0.2	0.3	0.04	50	65-55*	224
	18	1X	2	0.15	0.2	0.3	0.04	50	50º	266
	19	1X	2	0.15	0.2	0.3	0.04	50	65-55*	221
	20	1X	2	0.15	0.2	0.3	0.04	50	65-55*	181
	21	1X	2	0.15	0.16	0.3	0.02	50	55º	435
	22	1X	2	0.15	0.2	0.3	0.04	50	65-55*	297
	23	1X	0,8	0.16	0.64	-	0.01	50	58º	252
	24	1X	2	0.15	0.32	0.3	0.02	50	55º	549
	25	1.25X	2.5	0.19	0.25	0.4	0.05	50	65-55*	203
	26	1.25X	2.5	0.19	0.25	0.4	0.05	50	65-55*	315
RSPO4	1	1X	1	0.2	1	-	0.01	20	54º	407
	2	1X	1	0.2	0.5	-	0.01	50	50º	201
	3	1X	1	0.2	0.5	-	0.01	50	50º	243
	4	1X	1	0.2	0.5	-	0.01	50	50º	243
	5	1X	1	0.2	0.5	-	0.01	50	50º	282
LEMD3	13	1X	3	0.15	0.4	0.3	0.05	25	65-55*	400
ENPP1	4	1X	3	0.15	0.2	0.14	0.05	50	60º	396
PPP2R2D	7	1X	1	0..2	0.5	-	0.01	50	60	363

Gene	Exon	PCR conditions							At (ºC)	bp
		Buffer	MgCl2 (mM)	dNTPs (mM)	Primers (F+R) pmol	BSA (mg/ml)	GoTaq (U)	DNA (ng)		
PLCG2	19	1X	1	0.16	1	-	0.01	30	55º	257
	21	1X	1	0.16	1	-	0.01	30	55º	584
	33	1X	1	0.16	1	-	0.01	30	55º	494
AMER3	3	1X	1	0.2	1	0.4	0.01	30	65-55*	233
FLNC	26	1X	1.25	0.2	1	-	0.03	25	65-55*	434
FGF2	3´UTR	1X	1.5	0.2	0.5	-	0.01	50	52	135
COL11A2	6	1X	1.25	0.2	0.5	-	0.01	50	60	170
VDR	5	1X	1	0.2	0.5	-	0.01	50	60	133
BMP4	7	1X	1	0.2	1	-	0.01	50	60	170
BMP2	3	1X	1.5	0.2	0.5	-	0.01	50	52	167
ABCC6	5'UTR-LR	1X	1.5	0.2	0.5	-	0.03	50	68	1929
	2	1X	1.25	0.2	0.5	-	0.03	20	66	311
	3-4-LR	1X	1.25	0.2	0.5	-	0.03	20	68	2356
	5	1X	1.25	0.2	0.5	-	0.03	20	62	295
	6	1X	1.25	0.2	0.5	-	0.03	20	62	194
	7	1X	1.25	0.2	0.5	-	0.03	20	62	259
ABCC6	8-9-LR	1x		0.4	1	-	0.04*	40	68	5626
	10-LR	1X	1.25	0.2	0.5	-	0.03	20	64	1193
	11	1X	1.25	0.2	0.5	-	0.03	20	64	253
	12	1X	1.25	0.2	0.5	-	0.03	20	64	387
	13	1X	1.25	0.2	0.5	-	0.03	20	64	286
	14	1X	1.25	0.2	0.5	-	0.03	20	64	262
	15	1X	1.25	0.2	0.5	-	0.03	20	64	219
	16	1X	1.25	0.2	0.5	-	0.03	20	66	282
	17	1X	1.25	0.2	0.5	-	0.03	20	62	343
	18	1X	1.25	0.2	0.5	-	0.03	20	64	318
	19	1X	1.25	0.2	0.5	-	0.03	20	66	374
	20	1X	1.25	0.2	0.5	-	0.03	20	64	221
	21	1X	1.25	0.2	0.5	-	0.03	20	63	305
	22	1X	1.25	0.2	0.5	-	0.03	20	66	431
	23	1X	1.25	0.2	0.5	-	0.03	25	68	533
	24	1X	1.25	0.2	0.5	-	0.03	20	62	322
	25	1X	1.25	0.2	0.5	-	0.03	20	62	270
	26	1.25X	1.25	0.12	1	-	0.03	50	62	258
	27	1X	1.25	0.2	0.5	-	0.03	20	65	297
	28	1X	1.25	0.2	0.5	-	0.03	20	66	371
	29	1X	1.25	0.2	0.5	-	0.03	20	66	296
	30	1X	1.25	0.2	0.5	-	0.03	20	66	344
	31	1X	1.25	0.2	0.5	-	0.03	20	66	270

Abbreviations: DMSO: Dimethyl sulfoxide, BSA: Bovine Serum Albumin, At: Annealing temperature, F: forward, R: reverse, bp: base-pair. * Long and accurate (LA) taq (TAKARA).

3.2.1.3 Sequencing

The PCR products with a positive band were then purified using ExoSAP-IT™ (Exonuclease I and Shrimp Alkaline Phosphatase) according to manufactures instructions. Sequencing reaction with BigDye® Terminator v3.1 and v1.1 was performed in a 10 µl volume containing 2 µl template, 2 µl 5X Sequencing Buffer, 1 µl Sequencing Premix (Big Dye v3.1 or v1.1), 0.16 µl primer (10pmol) (Table 3-9) and ddH_2O to achieve final

volume. Reaction conditions included an initial denaturation step at 96ºC for 1 minute followed by 25 cycles of: denaturing at 96◦C for 10 seconds, annealing at 50◦C for 5 seconds, and elongation at 60◦C for 4 minutes. Purification of the products for sequencing was performed by precipitating with 0.5 M EDTA and 3 M sodium acetate, and a final precipitation with 70% and absolute EtOH. Templates were ressuspended in 15 µl highly deionized formamide (HiDi formamide) and sequenced using the ABI 3130xl (Applied Biosystems®).

3.2.1.4 Sequence analysis

Genetic variants of exons and intron-exon boundaries of the genes were screened with Sequencing Analysis and SeqScape software (Applied Biosystems®) using as reference the sequences present in NCBI (Table 3-11).

Table 3-11. NCBI references for the genes studied.

Gene	NCBI reference
SLC12A3	NG_009386.1
RSPO4	NG_013043.1
LEMD3	NG_016210.1
ENPP1	NG_008206
PPP2R2D	NM_018461.4
PLCG2	NG_032019.2
AMER3	NM_152698.2
FLNC	NG_011807.1
BMP4	NG_009215.1
FGF2	NG_029067.1
BMP2	NG_023233.1
VDR	NG_008731.1
COL11A2	NG_011589.1
ABCC6	NG_007558.2

3.2.2 Tissue expression

3.2.2.1 Cartilage homogenization

Cartilage sections preserved in RNA later were reduced to a powder using a mortar and pestle and liquid nitrogen. The cartilage powder was placed in 1 ml TRIzol reagent and stored at -80ºC for 24 hours.

3.2.2.2 RNA isolation

The RNA was obtained using a TRIzol RNA isolation protocol. Trizol reagent (Invitrogen) is a ready to use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate is an improvement of the single-step RNA isolation method development by Chomezynski and Sacchi [310]. During sample homogenization or lysis, TRIzol reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation was used to separate the aqueous phase containing RNA and the organic phase containing DNA and other material.

- **Separation phase**

- The samples were thawed by placing them at room temperature for 10 minutes;
- Placed into the Thermo Mixer at 37˚C for 10 minutes and 1400 rotations per minute (r.p.m);
- 0.2 ml of chloroform was added and the tubes vortexed for 15 minutes at the highest velocity;
- Samples were then centrifuged for 10 minutes at 8◦C at 10600 r.p.m..

Note: Following centrifugation, the mixture separates into different phases: a lower red, phenol-chloroform phase, an interface, and a colourless aqueous phase. RNA remains exclusively in the aqueous phase.

- **RNA precipitation**

- The aqueous phase was transferred to a new RNase free tube (approximately 0.5 ml);
- The washing was repeated with chloroform, but it was vortex only 15 seconds, centrifuged for 10 minutes at 8˚C at 10600 r.p.m and the aqueous phase was transferred to a new RNase free tube;
- The RNA was precipitated from the aqueous phase by mixing with 0.5 ml of cold (4◦C) 100% EtOH. The tube was inverted 5 times to mix;
- The samples were incubated at -20ºC for 30 minutes and were centrifuged at 10 minutes at 10600 r.p.m.

Note: The RNA precipitate forms a gel-like pellet on the side and bottom of the tube.

- **RNA wash**

- The supernatant was removed by inverting the tube and the RNA pellet washed with 1 ml of 75% EtOH;
- The samples were mixed by inverting the tube. The samples were centrifuged at no more than 8400 r.p.m for 5 minutes at 8◦C;
- The supernatant was taken off carefully with a 1000 µl pipet (always at the opposite side of the pellet).

- **Redissolving the RNA**

- The RNA pellet was dried in a Thermo Mixer at 37ºC for 2-5 minutes; it is important not to let the RNA pellet dry completely as this will significantly decrease its solubility;
- The RNA was dissolved immediately in 24 µl RNase-free water.

3.2.2.3 RNA purification

3.2.2.3.1 RNA clean-up

The clean-up of the RNA samples was performed using the RNeasy MinElute Cleanup kit (Qiagen) according to the manufacturer's instructions. This clean-up protocol is important for RNA to be used for enzymatic reactions, for desalting, and for concentrating the RNA.

3.2.2.3.2 DNase digestion

All the samples were digested with DNase (Deoxyribonuclease I). We added to the RNA samples 10 µl of the DNA digestion buffer RDD (RNase-Free DNase Set) and 2,5 µl of DNase solution and the volume was completed with water up to 100 µl. The samples were placed into the Thermo Mixer at 23°C for 10 minutes. Subsequently the samples were cleaned using RNeasy MinElute Cleanup kit (Qiagen) adding β-mercaptoethanol to RLT buffer (RNeasy Lysis Buffer) to obtain better results.

3.2.2.3.3 Ethanol precipitation of RNA

For the samples with poor quality we used 3 EtOH wash. We added to the RNA sample 1/10 volume of Sodium Acetate (3 M, pH 5.2) and 3 x volume of 100% EtOH. The samples were incubated on ice 15 minutes and centrifuged at 14,500xg for 30 minutes at room temperature. The supernatant was discarded and the pellet washed with 70% EtOH. The samples were centrifuged again for 15 minutes at the same speed. The supernatant was discarded and the pellet was dry at room temperature to evaporate all the EtOH. The pellet was dissolved in 14µl RNase free water.

3.2.2.4. RNA quality assessment

3.2.2.4.1 Concentration and integrity

For detection of RNA quality, RNA yield, A260:A280 ratio (good ratios between 1.8 and 2.0) and concentration were determined using a NanoVue spectrophotometer (GE Healthcare). RNA integrity was determined by measuring 28S/18S rRNA ratios and calculating the RNA integrity number (RIN) using an Agilent 2100 Bioanalyser and RNA 6000 Nano LabChip (Agilent Technologies).

3.2.2.4.2 Reverse transcription polymerase chain reaction (RT-PCR) and β-Actin test

The RNA samples with RIN numbers above 4 were used for cDNA synthesis using 100 ng of total RNA and a High Capacity cDNA Reverse Transcription kit and following the manufactures instructions.

PCR amplification of a fragment of β-actin gene, the reference gene, was performed in a 20 µl final reaction volume using the following conditions: 1X GoTaq Flexi Buffer; 0.125 mM of each dNTP; 0.8 mM of MgCl; 5 pmol of each primer and 0.25 U of GoTaq (Promega). 100 ng of cDNA was used in each reaction and deionized water was used to attain the final volume. Primer sequences and optimal annealing temperatures applied are given in table 3-12.

Table 3-12. Forward and reverse oligonucleotide primers (5'→3') used in PCR reactions of β-Actin gene.

Forward	Reverse	At (ºC)	PCR band size (bp)
TGGTGGGCATGGGTCAGA	GTACATGGCTGGGGTGTTGA	54	249

Abbreviations: At- Annealing temperature, bp- base-pair.

3.2.2.5 Gene Expression

The Real-Time PCR reactions were performed with TaqMan chemistry using an Applied Biosystems 7500 Fast Real-Time PCR System. For each sample duplicate reactions were used and the TaqMan Gene Expression Assays: Hs00184566_m1 for the target gene (*ABCC6* gene) and the Hs00237047_m1 for the endogenous control gene *YWHAZ* (Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta). The reactions were made using 200 ng of cDNA in the control assay plate and 400ng in the target gene plate. The PCR conditions were performed according to the manufacturer's instructions. For each assay we used Standard run and the comparative Ct (cycle threshold) method. The data analysis was performed using the comparative ΔCt method (ΔCt).

CHAPTER IV: CHONDROCALCINOSIS ASSOCIATED WITH GITELMAN SYNDROME

4. CHONDROCALCINOSIS ASSOCIATED WITH GITELMAN SYNDROME

4.1 Abstract

Gitelman Syndrome (GS) is a rare autossomal recessive inherited tubulopathy caused by mutations in the *SLC12A3* gene. The association of GS with chondrocalcinosis (CC) has been described in the literature as a typical example of hypomagnesemia-induced crystal deposition disease but its role in CC development is not fully understood. We aimed to investigate the association between GS and CC, by analysing one Azorean kindred, with an index-case presenting CC, hypomagnesemia and hypokalemia.

SLC12A3 gene was screened in the proband and the variant detected was procured in family members. The proband was homozygous for the S615L mutation and presented chondrocalcinosis. Seven of the tested individuals in the probands family were heterozygous for the mutation and one presented CC. The presence of CC in two other individuals of the family was most likely sporadic, and associated with their advanced age.

The genetic cause for GS in a proband with secondary early onset CC was associated with S615L mutation of the *SLC12A3* gene.

Keywords: *SLC12A3*, Gitelman syndrome, Chondrocalcinosis, hypomagnesemia.

4.2 Introduction

Gitelman Syndrome (GS, OMIM #263800) is a rare autossomal recessive tubulopathy with a prevalence of approximately 1:40.000 in the Caucasian population [311]. Onset usually occurs in adult life, but cases of childhood onset are also known [311]. The condition is characterized by hypomagnesemia, hypokalemia, metabolic alkalosis, hypocalciuria and hyperreninemic hyperaldosteronism [312]. The clinical spectrum is wide and includes: cramps, myalgies, muscle weakness, tetany, and paralysis [313]. GS is caused by inactivating mutations in member 3 of the solute carrier family 12 gene (*SLC12A3*), which consists of 26 exons and is located on the long arm of chromosome 16 [311]. This gene encodes a thiazide-sensitive sodium-chloride cotransporter (NCCT) expressed in the distal convoluted tubule of the kidney. NCCT is a polypeptide which consists of 1021 amino acids. Its 2-D structure is predicted to contain 12 transmembrane domains and intracellular amino- and carboxyterminal regions [313, 314]. To date, according to the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/), more than 300 different mutations have been identified in patients with GS. Detected mutations include missense, non-sense, frame-shift, and splice-site mutations and are scattered throughout the transporter protein with a possible clustering of mutations in the carboxy-terminal tail [315, 316]. Genetic heterogeneity exist, and a minority of patients with GS phenotype, who do not have mutations in the *SLC12A3* gene, has been shown to have mutations in the *CLCNKB* gene [317, 318]. This gene encodes the renal chloride channel ClC-Kb, located in the basolateral membrane of cells of the thick ascending limb of Henle's loop and the distal tubules. Mutations in *CLCNKB* gene have also been associated with classic Bartter syndrome, the most important genetic disorder to consider in the differential diagnosis of GS. Both loss-of-function mutations in NCCT and in CLC-Kb lead to disruption of NaCl reabsorption in the distal convoluted tubule. Therefore, it is important to screen the *CLCNKB* gene in patients with GS who do not have mutations in the *SLC12A3* gene [319].

The association of GS with CC has been described in the literature as a typical example of hypomagnesemia-induced crystal deposition disease [312]. CC is characterized by deposition of crystals of calcium pyrophosphate dihydrate (CPPD) in articular hyaline and fibro-cartilage [312]. Several cases of GS associated with CC have been published in the literature [261, 312, 320-331]. The role of hypomagnesaemia in the development of CC, however, is not fully understood. A cross-sectional study demonstrated that CC was

significantly higher in patients with lower serum magnesium levels (OR 13.5, 95% CI 2.76-127.3, P<0.0001) [332]. Magnesium is an important cofactor for alkaline pyrophosphatase, an enzyme that plays a key role by converting inorganic pyrophosphate (PPi) to orthophosphate (Pi). A reduction in the activity of this enzyme due to hypomagnesaemia could induce CPPD by raising extracellular levels of PPi that with calcium is a crucial precursor for CCPD crystal nucleation [333]. CPPD may be found in other conditions associated with hypomagnesaemia, such as short bowel syndrome or tacrolimus therapy in liver transplantation patients [333].

The aim of this study was to investigate the association between GS and CC, by analysing one Azorean kindred, with an index-case presenting CC, hypomagnesemia and hypokalemia. The *SLC12A3* gene was screened in the proband and the variant detected was procured in family members and they were also clinically evaluated to determine if they presented CC.

4.3 Material and methods

The proband, a 60 years-old Caucasian male, was first observed in the Rheumatic Diseases Clinic, Hospital de Santo Espírito da Ilha Terceira (HSEIT), Azores, Portugal when he was 48 years old. The proband and his parents were born in Terceira island. Symptoms started when the proband was 33 years old, mainly affecting knees, ankles, wrists, elbows and achilles tendons. In the proband, pyrophosphate crystals were identified in the synovial fluid aspirated from a right knee effusion. From that time he was treated with colchicine, NSAIDS (Nonsteroidal anti-inflammatory drugs), and oral potassium and magnesium. Laboratory tests revealed normal leukocyte, erythrocyte and platelet count. Blood urea was 33 mg/dl, creatinine 0.9 mg/dl and glucose 177 mg/dl. Serum electrolyte concentrations were as follows: sodium 139 mEq/L, potassium 3.2 mEq/L, calcium 9.8 mg/dl, and magnesium 1.1 mg/dl. In spite of the treatment with colchicine, patient remained hypokalemic and hypomagnesemic, however he showed some improvements. Using the diagnostic criteria of Bettinelli et al [334] a clinical diagnosis of GS was suspected, and a diagnosis of knee CC was made after the identification of bilateral knee cartilage calcification (Figure 4-1). The family members of the proband were investigated and a blood sample and x-rays were taken from all of them.

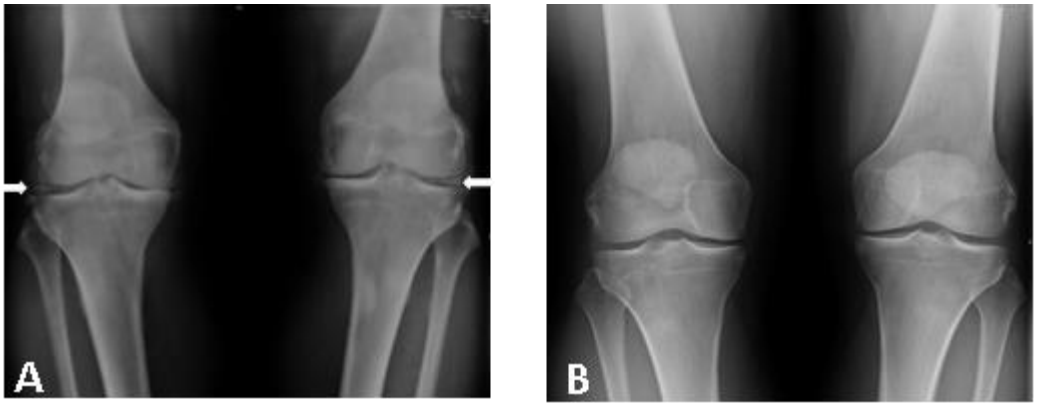


Figure 4-1. X-rays of proband showing classical CC in knees (image A) and his son without CC (image B). The presence of CC is indicated by arrows.

Genomic DNA was extracted from peripheral blood cells using the salting out procedure and used as the template for PCR amplification of individual exons of the *SLC12A3* gene. Twenty-six pairs of oligonucleotide primers were generated to amplify all 26 exons (primer sequences and PCR conditions available in chapter 2 of this thesis).

PCR products were purified using ExoSAP-IT™ and following the manufactures instruction and then sequenced using BigDye® Terminator v3.1. In brief, PCR amplicons were ressuspended in 15 µl formamide and directly sequenced using the ABI 3130xl sequencer (Applied Biosystems®). Genetic variants of all the 26 exons and intron-exon boundaries of the *SLC12A3* gene were screened with SeqScape (Applied Biosystems®) using as reference the NCBI sequence NG_009386.1. *SLC12A3* gene was screened in the proband and the variant detected was then screened in family members. This study was approved by the HSEIT Ethics Committee and all participants provided informed consent.

4.4 Results

Through *SLC12A3* direct sequence analysis in the proband a silent variant, A to G transition at position c.366 (rs2304479) in exon 2 and a missense substitution in exon 15 (rs779160677) previously described as associated to GS were detected [335]. This mutation (rs779160677), a C to T transition at position c.1869, changed the small size and polar amino acid serine to a medium size and hydrophobic leucine at position 615 (S615L), and had a SIFT score of 0 and a PolyPhen value of 0.996, both values suggestive of a deleterious variation.

The presence of the mutation in thirteen family members was investigated (5 men, 8 women; 25-79 years; mean age 51 years) and blood tests and x-rays were obtained from

each family member (data not shown). The pedigree with investigated individuals is shown in figure 4-2.

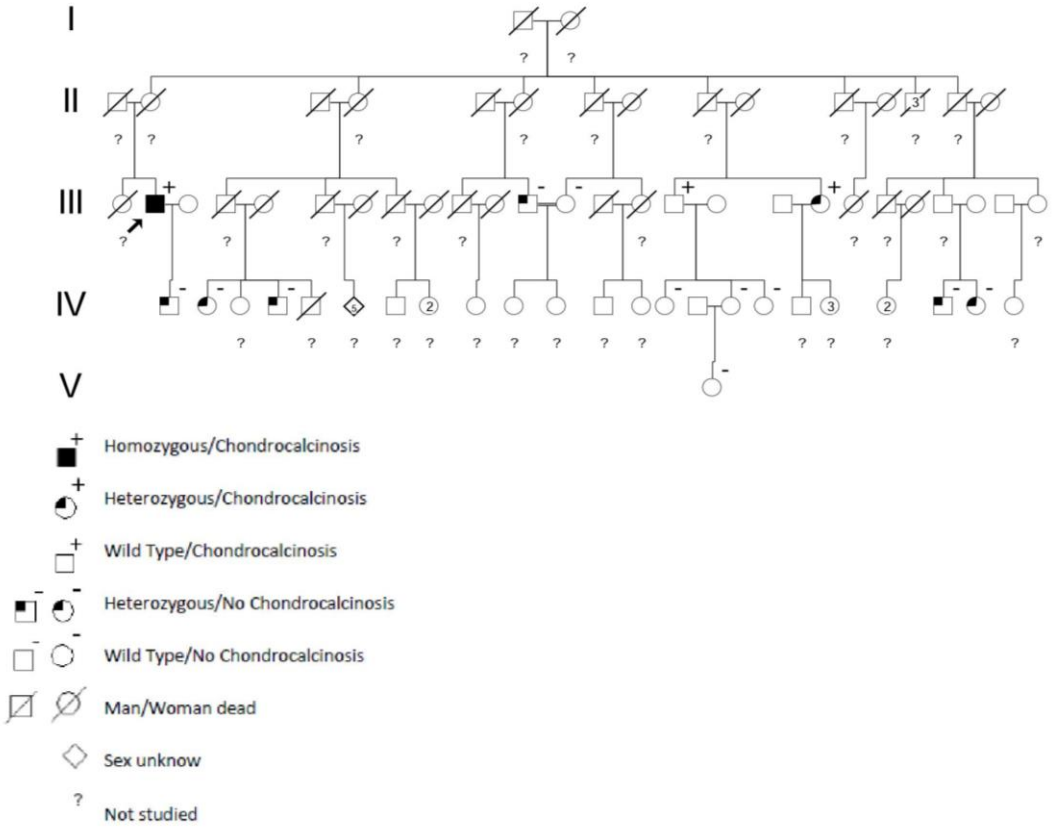


Figure 4-2. Heredogram with investigated individuals; Proband with GS and CC is indicated by the arrow.

Biochemical data in these patients revealed they had normal levels of serum magnesium ranging from 1.9 to 2.3 mg/dL and normal levels of potassium ranging from 3.7 to 4.7 mmol/L. Taking in consideration the ratio K/Mg, we found that all the individuals with CC (III.2, III.16 and III.19) had a racio >2.3. The only individual with a K/Mg ratio > 2.3 that did not present any signs of CC, is a Human leukocyte antigen (HLA) B27+ young female of only 25 years of age (Table 4-1).

Table 4-1. Characteristics and *SLC12A3* gene variants and blood chemistry levels in the proband and the thirteen selected individuals from their family pedigree. The proband is indicated by bold. a Normal serum magnesium 1.5 - 2.5 mg/Dl : b Normal serum potassium 3.3 - 5.1 mmol/L.

Individuals	Sex	Age	Mg [a] mg/dL	K [b] mmol/L	Racio (K/Mg)	CC	*SLC12A3* variants	
							A122A	S615L
III.2	M	62	1.1	**3.2**	2.9	+	A122A/A122A	S615L/S615L
III.12	M	75	2.1	4.7	2.23	-	A122A/WT	S615L/WT
III.13	F	67	2.2	4.1	1.86	-	WT/WT	WT/WT
III.16	M	75	2.0	4.6	2.3	+	WT/WT	WT/WT
III.19	F	79	1.9	4.7	2.47	+	A122A/WT	S615L/WT
IV.1	M	35	2.3	3.7	1.6	-	A122A/WT	S615L/WT
IV.2	F	54	2.2	4.2	1.9	-	A122A/WT	S615L/WT
IV.4	M	46	1.9	4.0	2.1	-	A122A/WT	S615L/WT
IV.14	F	51	1.9	4.0	2.1	-	WT/WT	WT/WT
IV.16	F	49	2.2	4.1	1.86	-	WT/WT	WT/WT
IV.17	F	45	1.9	4.3	2.26	-	WT/WT	WT/WT
IV.21	M	37	2.1	4.1	1.95	-	A122A/WT	S615L/WT
IV.22	F	36	2.0	3.7	1.85	-	A122A/WT	S615L/WT
V.1	F	25	2.0	4.7	2.35	-	WT/WT	WT/WT

Abbreviations: M- male, F- female, CC- Chondrocalcinosis, WT- wild type, Mg- Magnesium, K- potassium.

Seven of the 13 family members analysed were found to be heterozygous for the S615L mutation, but only one, a female of 79 years of age, presented CC. Furthermore, six individuals of the 13 were wild-type homozygous at position 615 in the gene, nonetheless, one of them, a male of 75 years of age (III.16) presented CC (Figure 4-2 and table 4-1).

4.5 Discussion

The GS patient described in this study has the S615L variation in homozygosity, while all of the other cases of GS with this variation were reported in compound heterozygote's (2, 10). In our study, seven individuals heterozygous for the S615L mutation did not have either hypokalemia or hypomagnesemia, indicating that they were asymptomatic carriers of this mutation. Hypomagnesemia and hypocalciuria are found in most cases of GS, however, some cases with mutations in the NCCT do not show these conditions [336]. It is believed that hypomagnesaemia causes CC by increasing the formation and at the same time reducing the solubility of CCP crystals [333]. It is known that an excess of PPi is the main precursor for CPP crystal nucleation. Because magnesium is a necessary cofactor

for numerous enzymes, such as pyrophosphatases, and considering the fact that it increases the solubility of CPP crystals, it has been proposed that low levels of magnesium could induce CPP deposition disease by raising PPi and/or reducing the saturation product of CPP crystals [329].As far as we know the ratio Mg/K was never considered in CC development. In this study, this ratio seems relevant since all the patients with CC show a ratio >2.3. Individual V.1, presenting a K/Mg ratio of 2.35 does not show any signs of CC yet, however she is HLA-B27+ (B*2705;49). HLA-B*27 belongs to the MHC complex on chromosome 6 that is strongly associated with ankylosing spondylitis, a disease characterized by the presence of calcifications on the axial skeleton [131, 132].

The prevalence of CC increases with age (10-15% for people between 65 and 75 years) and is hence called sporadic in patients older than 60 years, whereas in younger individuals there are several putative underlying disorders causing CPPD deposition disease, such as hemochromatosis, hyperparathyroidism, hypomagnesemia or hypophosphatemia [337]. The assumption that GS is caused by a defect in the NCCT cotransporter in the renal distal tubule has been demonstrated by the association of a number of different mutations in the *SLC12A3* gene in patients with GS [311, 315, 336, 338]. In spite of the growing number of causative mutations identified in GS patients, more than 40% of patients carry only a single mutation in *SLC12A3*, instead of being compound heterozygous or homozygous, suggesting mutations may predispose to the disease and in the presence of other factors, yet to be identified, the disease develops or not [3].

In the present study, the specific involvement of this cotransporter in the aetiology of this disorder is further substantiated by the finding that the proband is homozygous for the S615L variation. The S615L identified in this study has previously been described by Cruz and co-workers [335] in a study involved 36 kindreds from the United States, Canada and England and later reported in a study by Syrén *et al* [339] in which 21 patients from 19 unrelated families were investigated and fifteen new mutations were identified. Although the *SLC12A3* variations reported in previous studies are distributed throughout the whole protein [315, 340], the study of Lemmink et al [315] indicates that the carboxy-terminal end represents a hot spot for variations. S615L is located at the intracellular C-terminal end of the NCCT protein. It is conceivable that structural alterations due to *SLC12A3* variations in the C-terminal domain interfere with

phosphorylation of the NCCT protein and as such with its regulation and that this creates physiological conditions that favour CCP crystal formation [315]. Evidence for an association between CC and GS mainly comes from uncontrolled case reports, case series and only one cross-sectional study. As a result, its epidemiology remains unknown [333]. There have been few cases described with a definite diagnosis of CC due to GS. In some patients with CPPD deposition disease secondary to hypomagnesemia, the stabilization of magnesium and potassium levels can reduce the deposition of CPP crystals in the synovium and synovial fluid, reducing the frequency of attacks of articular pain [329].
We are facing a case of a pedigree where the genetic cause for GS has been identified. The presence of CC in two individuals of this family is probably sporadic since they are both older than 65 years old. In this study, the number of patients included was small (one family); however our results suggest that our proband had an early onset of CC because it was secondary to GS. Further studies are needed in order to gain insight into the pathophysiology and prevalence of CC in patients with GS.

4.6 Conclusion

GS is a hereditary disease characterized by defective tubular reabsorption of magnesium and potassium, mostly caused by mutations in the *SLC12A3* gene. Sometimes GS patients, as in our study, might come with a CC diagnosis. We identified the genetic cause for GS in a proband with secondary early onset CC. Further studies are needed in order to shed light on the pathophysiology and prevalence of CC in patients with GS.

4.7 Future work

1) Typing the HLA-B-allele of all family members to verify from where derives the $B27^{+}$
2) Verify the relevance of Mg/K ratio in one cohort of CC patients comparing with a control group.

CHAPTER V:
INVESTIGATING THE ROLE OF THE *RSPO4* AND *LEMD3* GENES WITH DISH/CC PHENOTYPE

5. INVESTIGATING THE ROLE OF THE *RSPO4* AND *LEMD3* GENES WITH DISH/CC PHENOTYPE

5.1 Abstract

DISH/CC is a poorly understood phenotype characterised by peripheral and axial enthesopathic calcifications, frequently fulfilling the radiological criteria for DISH, and in some cases associated with CPPD Chondrocalcinosis (CC). The concurrence of DISH and CC suggests a shared pathogenic mechanism. To date, the *ANKH* gene is the only monogenic cause identified for CC and *COL6A1* and *FGF2* are two susceptibility genes with a weak genetic link to DISH. Two genes; *RSPO4* and *LEMD3* were identified in a previous analysis of shared chromosomal segments across 4 DISH/CC families from Terceira Island. The current study aims to investigate the possible link between *RSPO4* and *LEMD3* genetic variants in the aetiology of DISH/CC.

DNA from 55 patients with DISH/CC and 36 controls without DISH/CC were obtained for a case control association study. To verify the segregation within families, 74 family members from 9 families harbouring one or more genetic variant in *RSPO4* or *LEMD3* genes were investigated. The entire DNA coding region of the candidate gene *RSPO4* and exon 13 of the *LEMD3* gene were amplified by PCR and Sanger sequencing and statistical analysis was performed using Plink V1.9.

Nine genetic variants were identified in the *RSPO4* gene; 3 regulatory region variants (rs146447064, rs149154047 and rs6056520), 1 splice site variant (rs775644973), 2 synonymous (rs150446609 and rs41275604) and 3 missense mutation variants (rs6140807, rs201485021 and rs61740632). No statistically significant difference in the occurrence of these genetic variants was observed in DISH/CC phenotype relative to the control. However, two regulatory variants (rs146447064 and rs14915407) are significantly more frequent in controls than in DISH/CC patients. The 10 genetic variants in *RSPO4* and in *LEMD3,* did not segregate within the families studied.

The results of the present study revealed that the *RSPO4* gene regulatory variants: rs146447064 rs149154047, may protect against the DISH/CC phenotype in Terceira Island, possibly by altering gene expression of the *RSPO4* gene. Variant rs201930700 in *LEMD3* is extremely rare thus, its effect is difficult to ascertain at this point.

Keywords: *RSPO4*, *LEMD3,* Chondrocalcinosis, DISH, sequencing, variants.

5.2 Introduction

Diffuse Idiopathic Skeletal Hyperostosis (DISH, MIM 106400) and Chondrocalcinosis (CC; MIM #118600) are diseases characterized by ectopic calcifications. DISH is characterized by the ossification of enthesis in the axial and peripheral skeleton, affecting the anterior longitudinal ligament, in particular the right side of the spine, with preservation of the intervertebral disc space [1]. CC is characterized by the deposition of calcium containing crystals in articular cartilage, synovial membranes and, less often, in periarticular soft tissues [341, 342]. *ANKH* mutations are the only known cause for a very small number of cases of monogenic CC (CC; MIM #118600) [110, 343-345] as well as craniometaphyseal dysplasia (CMDD; MIM #123000) [346-348]. The *ANKH* gene maps on chromosome 5 (5p15.1) and encodes a multipass transmembrane protein ANK that transports intracellular PPi to the extracellular milieu [106], where it acts as a potent inhibitor of mineralization [108]. The aetiology of DISH is still unknown, but several lines of evidence suggest that genetic factors might be involved in its aetiology [64, 77, 78]. Very few genetic studies on DISH have been published and up until now only two genes - *COL6A1* [33, 146] and *FGF2* [147] - have been shown to have a positive association with DISH susceptibility. Nonetheless, variants that showed significant association, in both genes, are located in non-coding regions and are very common variants within the general population, which suggests that these variants have only a minor effect on DISH susceptibility. DISH is very similar to, and can coexist with Ossification of the Posterior Longitudinal Ligament (OPLL; MIM 602475), a disease in which the genetic background is considered relevant in its aetiology. Unlike DISH, OPLL has been extensively investigated and despite some conflicting studies, a great number of susceptibility genes have been reported along the years. These included genes for Collagen 6A1 and 11A2 (*COL6A1* and *COL11A2*) [33, 177], Bone morphogenetic protein 2 and 4 (*BMP2* and *BMP4*) [158, 161], Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 (*ENPP1*) [166], Transforming growth factor 1 and 3 receptor (*TGFβ1* and *TGFβ3*) [154, 170], Estrogen receptor 1 (*ESR1*) [154], R-Spondin 2 (*RSPO2*) [172, 349] among others. However, as in DISH, genetic variants with a positive association with OPLL seem to have a minor effect on susceptibility to the disease.

The coexistence of DISH with CC is very common on Terceira Island-Azores leading our group to hypothesise that both diseases, hereafter designated as DISH/CC phenotype,

share the same pathogenic mechanism [23]. A similar phenotype has been reported in several studies in the past [25, 26]. In a previous study, our group, try to determine the genetic cause for this phenotype using a whole genome linkage analysis followed by an "Identity-by-state/descent" mapping in 10 affected individuals from 4 different families. Identity by descent (IBD) mapping is a statistical method for detection of genetic loci that share an ancestral segment among "unrelated" affected pairs of individuals. IBD mapping is more robust to allelic heterogeneity and can be used as a complementary method to genome-wide linkage studies, to identify rare inherited variants when combined with sequence data. The chromosome zones considered of interest were selected taking into consideration the maximum pairs sharing. Two zones, in chromosome 12 (65667554 – 68670915) and 20 (821749-1266214 and 5157217-6074302) had the maximum number of pairs and thus the genes within this region were investigated. Two candidate genes were further investigated: *RSPO4* in chromosome 20 (Chr 20:958452 – 1002284) and *LEMD3* located very near the chromosome 12 (65169571-652483279). *RSPO4* encodes a member of the R-spondin family, a group of four proteins which positively regulate canonical Wnt signaling by reducing Wnt receptor turnover and thereby increasing beta-catenin stabilization. R-spondins may contribute to the maintenance of adult bone mass by regulating osteoblastogenesis and bone formation [350]. Loss of function mutations in the *RSPO4* gene cause congenital anonychia (NDNC4; MIM #206800) or the absence of nails [351]. *LEMD3* (LEM Domain Containing 3) encodes the Inner nuclear membrane protein Man1, which helps to control two important signaling pathways namely the Transforming Growth Factor-βeta (TGF-β) and the Bone Morphogenetic Protein (BMP) signaling. Man1 can interact directly with the TGF-β superfamily ligands, including bone morphogenic proteins (BMPs) and activin, or via its C-terminal domain, directly with Smad, which bind to specific areas of DNA to activate specific genes [352]. Genetic variants of *LEMD3* have been associated with Osteopoikilosis and Buschke-Ollendorff Syndrome (BOS; MIM #166700) [353]. The present study targets genetic variants of the *RSPO4* and *LEMD3* genes located in chromosomal segments identified in a previous study as being associated with DISH/CC.

5.3 Material & methods

5.3.1 Subjects

This study involved nine DISH/CC families and studied 74 members (44 males; 30 females). Blood was obtained from all members who gave informed consent. Genomic DNA was extracted from peripheral blood cells using a standard Salting-out procedure. Standard X- rays were taken from: knees, axial skeleton, wrists, hands, elbows and pelvis. Amongst the 74 members, 46 were affected with DISH/CC, 20 had no signs of the disease and 8 were too young (< 25 years old) to establish likely disease status. A group of 55 unrelated Azorean patients with a diagnosis of DISH/CC (36 male, 19 female; age of onset around 40 years) and 36 unrelated controls (with no signs of DISH/CC) with a similar ethnic background (16 male, 20 female; mean current age, 68 years; range, 57-102) were also included.

The *LEMD3* rs201930700 frequency was evaluated on a randomized population of 124 individuals from Terceira Island (45 males, 79 females; mean age, 66 years; range, 35-100). This study was conducted with the approval of the HSEIT Ethics Committee.

5.3.2 *RSPO4* & *LEMD3* sequencing

RSPO4 gene primer pairs were designed using the software Primer3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) to amplify/sequence regulatory, coding regions and intron exon boundaries, from 55 patients with DISH/CC disease and from 36 unaffected controls subjects. The *LEMD3* variant (rs201930700) was typed using primers previously described by Hellemans and collaborators [307].

All primers, amplification conditions and codes for reference sequences are available in the materials and methods section of this thesis. PCR fragments were purified with ExoSAP-IT™ and sequenced using ABI Big Dye chemistry (unidirectional, or when necessary, bidirectional) followed by purification with EDTA/Sodium Acetate and ethanol precipitation. Sequencing products were run on an automated DNA sequencer ABI 3130XL (Applied Biosystems®) and genetic variants were screened by sequence analysis and SeqScape (Applied Biosystems®). Base calling for heterozygous positions was made if the lower peak of the two co-incident peaks was higher than 25% of the highest peak.

The genetic variants found in the *RSPO4* gene were screened within the families, where they occurred, whenever possible. The *LEMD3* rs201930700 variant was typed in a representative randomized group of 124 individuals from Terceira Island.

5.3.3 Statistical analysis

All SNPs were checked for Hardy-Weinberg equilibrium (HWE). For all DISH/CC families, a transmission disequilibrium test (TDT) was used to assess the difference of the allele frequencies between the affected patients with DISH/CC and unaffected individuals. The TDT test was calculated for CHISQ, odds ratios (OR) (95% confidence interval) and corresponding p-values. To assess the difference in allele frequencies between the 55 patients with DISH/CC and the 36 control individuals and between males and females a Fisher exact test was used. The Fisher exact test was calculated for odds ratios (OR) (95% confidence interval) and corresponding p-values.

To perform an association between the DISH/CC disease and an allele variant other tests were employed: Cohran-Armitage trend, dominant and recessive gene action tests with 1 degree of freedom and genotypic test with 2 degree of freedom. For all statistical tests used a p-value of ≤ 0.05 was considered statistically significant. All statistical analysis were performed using PLINK software [354].

5.4 Results

5.4.1 *RSPO4* sequencing

Nine genetic variants were identified in the *RSPO4* gene; 3 missense variants (rs6140807 and rs61740632), 1 splice site variant (rs775644973), 2 synonymous (rs150446609 and rs41275604) and 3 regulatory region variants (rs146447064, rs149154047 and rs6056520) (Table 5-1). All of them were in HWE. Two of the variants located on the regulatory region of the *RSPO4* gene (rs146447064 and rs149154047) were located in a fully conserved region and were rare, with a MAF value of 0.01 (Table 5-1). The same was observed for the synonymous variant rs150446609 which was very rare (<0.01) and was located in a fully conserved region. The missense variant rs201485021 was located in exon 3 and the SIFT score of 0 and PolyPhen value of 1 indicates that this variant has a deleterious and a damaging effect on the protein, respectively. In addition, this variant was located in a fully conserved region and was extremely rare (MAF <0.01); in the

"1000 genomes" (genomes from 26 different populations) the variant was identified in only 2 males from an Iberian population in Spain.

Table 5-1. Genetic variants identified in the *RSPO4* gene and functional significance information. Nucleotide conservation was obtained using an alignment of: Human, Chimpanzee, Mouse lemur, Pig, Hedgehog and Elephant available in the Ensembl database database (http://www.ensembl.org/index.html) (Accessed on January 2017).

Exon/ Intron	Variant	SNP	Type of variant	MAF	SIFT	PolyPhen	Nucleotide Conservation
Upstream gene	c.-131C>T	rs146447064	Regulatory region	**0.01**	NA	NA	Fully conserved
Upstream gene	c. -115G>A	rs149154047	Regulatory region	**0.01**	NA	NA	Fully conserved
5'prime	c. -85 G>A	rs6056520	Regulatory region	0.35	NA	NA	1 species non conserved (Mouse lemur)
Exon 1	c. 12 A>C	rs150446609	Synonymous p.Pro4=	**<0.01**	NA	NA	Fully conserved
Intron 1	c.79+1G>A	rs775644973	Splice donor (HGMD mutation)	**<0.01**	NA	NA	Fully conserved
Exon 3	c.317G>A	rs6140807	Missense p.Arg106Gln	0.03	0.21	0.072	2 species non conserved (Pig and Hedgehog)
	c.367 C>G	rs201485021	Missense p.Pro123Ala	**<0.01**	**0**	**1**	Fully conserved
Exon 4	c.471C>T	rs41275604	Synonymous p.Cys157=	0.01	NA	NA	1 species non conserved (Hedgehog)
Exon 4	c.524A>T	rs61740632	Missense p.His175Pro	0.01	0.3	0	4 species non conserved (Mouse lemur, Pig, Hedgehog and Elephant)

Abbreviations: SNP- Single nucleotide polymorphism, MAF- Minor allele frequency, SIFT- Sorting Intolerant From Tolerant, PolyPhen- Polymorphism Phenotyping, HGMD- Human gene mutation database, Pro- Proline, Arg- Arginine, Gln- glutamine, Ala- alanine, Cys- Cysteine, His- histidine, NA- not applicable.

In our study, this variant was found in only one female in our control group (n=36).The other two missense variants (rs6140807 and rs61740632), despite presenting SIFT and PolyPhen values indicatives of a minor effect on the protein (tolerated and benign, respectively), were both relatively rare. We found an extremely rare HGMD mutation (rs775644973 or CS065613), that has been associated with congenital anonychia [355]. The variant was found in only one female in our group of 55 DISH/CC patients, which is unaffected by congenital anonychia.

In order to perform a segregation analysis we tracked back the variants within 7 DISH/CC families, available in the AZORBIO biobank, where the variants were found. We investigated 7 variants: rs146447064 in AZ2, rs149154047 in AZ4, rs6056520 in AZ1, AZ2, AZ4 and AZ9, rs150446609 in AZ5, rs6140807 in AZ7 and AZ8, rs41275604 in AZ1 and AZ5 and rs61740632 in AZ1 and AZ7 families (Figure 5-1).

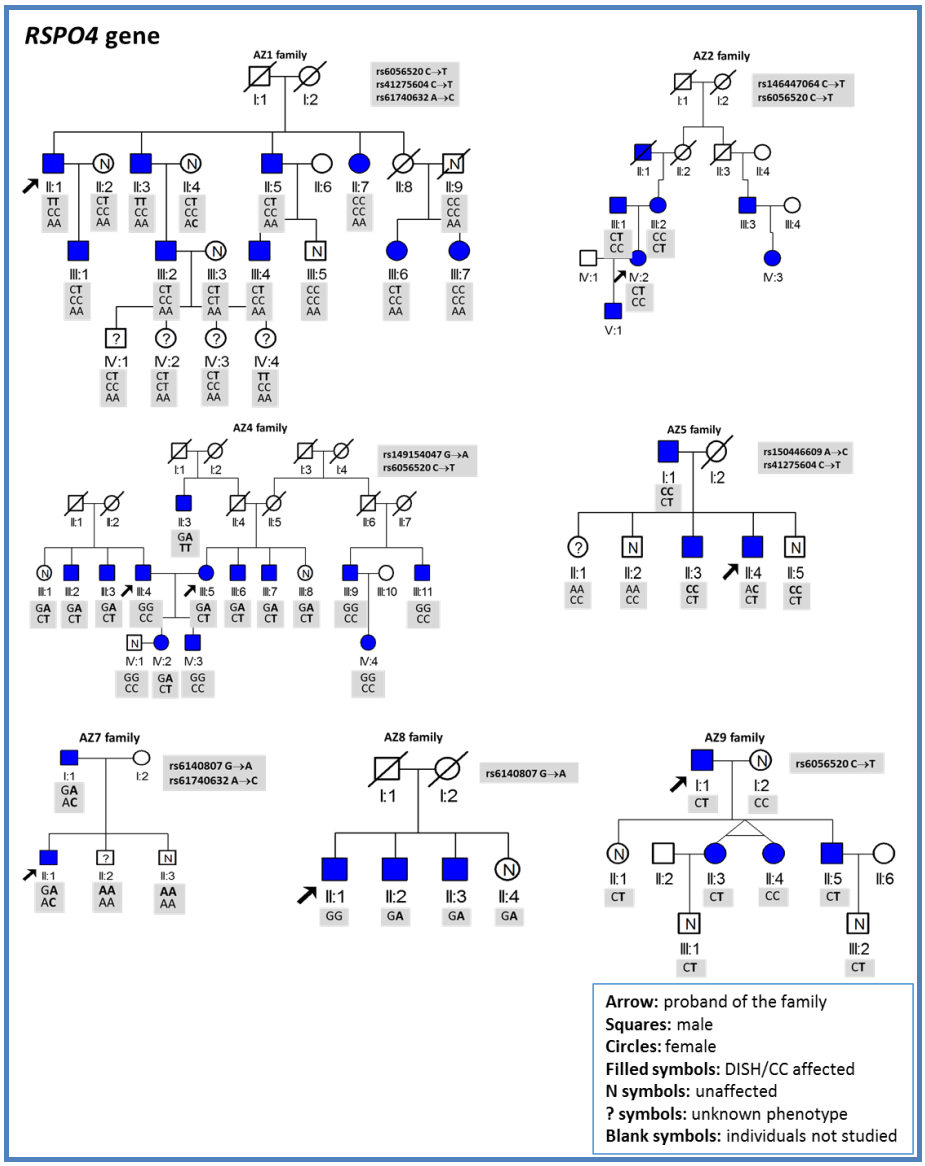


Figure 5-1. Typing results for *RSPO4* gene in DISH/CC families.

In AZ2 family we studied three individuals and we found the regulatory region variant **rs146447064** in heterozygosity in two of them (III:1 and IV:2) (Figure 5-1). In this family, all the individuals studied were affected and so the results from this family were not used for statistical tests.

The other regulatory region variant - **rs149154047 -** was found in heterozygosity in 9 individuals; 7 DISH/CC affected and in 2 unaffected. According to the statistical analysis shown in table 5-2, the variant rs149154047 was not associated with DISH/CC phenotype in AZ4 family. However, an important consideration in relation to this family was that of the 15 individuals studied, 12 of them are affected and only 3 were unaffected (individual III-1; III-8 and IV-1, with unknown age, 77 and 53 years, respectively).

Table 5-2. Family based association test (TDT test) results for variants of *RSPO4* gene.

SNP	Family	Alleles M/m	TDT			
			TR:UT	OR	CHISQ	P
rs146447064	AZ2	C/**T**	1:0	NA	1	0.317
rs149154047	AZ4	G/**A**	1:1	1	0	1
rs6056520	AZ1	T/**C**	0:2	0	2	0.157
	AZ2	C/**T**	0:1	0	1	0.317
	AZ4	C/**T**	1:1	1	0	1
	AZ9	C/**T**	2:1	2	0.333	0.564
rs61740632	AZ1	A/**C**	0:1	0	1	0.317

Abbreviations: SNP- Single nucleotide polymorphism, M/m – major allele/minor allele, TDT- Transmission disequilibrium test, TR:NT-Transmitted/Untransmitted minor allele account, OR- odds ratio, P- pvalue, p value, NA- not applicable.

The variant **rs6056520** was heterozygous in several families in a great number of individuals. However it was homozygous only in DISH/CC affected males of the families AZ1 and AZ4 (Figure 5-1).

The synonymous variant **rs150446609** in the AZ5 family was present in all DISH/CC affected individuals, however the variant was also found in one unaffected individual (II:5 individual) (Figure 5-1). In the AZ5 family few individuals were studied (6 individuals; 3 affected, 2 unaffected and 1 unknown), so it is not possible to draw great conclusions.

The missense variant **rs6140807** was present in all the individuals studied in AZ7 and AZ8 families, except the affected individual (proband) in AZ8 family (individual II:1) which was the wild type for this variant (Figure 5-1).

The synonymous variant **rs41275604** was found in AZ1 and AZ5 families. In the AZ5 family the 3 DISH/CC affected individuals were carriers of the rs41275604 variant, which contrasts with the AZ1 family in which none of the 9 DISH/CC affected individuals presented the variant. In this family the **rs41275604** variant was only found in

one unaffected female (III:3). No relationship was found between this variant and the DISH / CC phenotype.

The variant **rs61740632** in the AZ1 family was only found in one unaffected woman (II: 4), however segregation with the DISH/CC phenotype seemed to occur in the AZ4 family; the variant was present in all DISH/CC affected individuals and absent in the unaffected individuals. No association was verified maybe because the number of individuals studied was too small to reach statistical significance.

The *RSPO4* gene was further sequenced in 36 unrelated control individuals without DISH/CC in order to perform a case/control association study. The results are shown in table 5-3.

Table 5-3. Association study results of genetic variants found in *RSPO4* gene in Azorean patients with DISH/CC and controls without DISH/CC disease. The minor allele is represented in bold.

SNP	Alleles	MAF						Fisher exact test					
		DISH/CC			Controls			DISH/CC vs Controls					
		All N=55	F N=19	M N=36	All N=36	F N=22	M N=14	All		F		M	
								OR	P	OR	P	OR	P
rs146447064	C/**T**	0.05	0.03	0.06	0.14	0.18	0.07	0.30	0.03	0.12	0.03	0.76	0.67
rs149154047	G/**A**	0.05	0.07	0.03	0.10	0.07	0.14	0.44	0.22	1.17	1	0.17	0.05
rs6056520	C/**T**	0.28	0.18	0.33	0.19	0.18	0.21	1.63	0.22	1.02	1	1.83	0.33
rs150446609	A/**C**	0.05	0.05	0.04	0.01	0.02	0	3.38	0.41	2.39	0.59	NA	0.56
rs775644973	G/**A**	0.01	0.03	0	0	0	0	NA	1	NA	0.46	NA	1
rs6140807	G/**A**	0.18	0	0.03	0	0	0	NA	0.52	NA	1	NA	1
rs201485021	C/**G**	0	0	0	0.01	0.02	0	0	0.40	0	1	NA	1
rs41275604	C/**T**	0.04	0.05	0.03	0.03	0.05	0	1.32	1	1.17	1	NA	1
rs61740632	A/**C**	0.04	0	0.06	0.01	0.02	0	2.68	0.65	0	1	NA	0.57

Abbreviations: SNP- Single nucleotide polymorphism, MAF- Minor allele frequency, OR- odds ratio, P- pvalue, N- number of individuals, NA- not applicable, F- Female, M-Male.

The regulatory region variant **rs146447064** in heterozygosity was found in 9% of the DISH/CC patients and in 11% of the controls (Supplementary table 5-1). In homozygosity the variant was only found in the controls, particularly in 3 females

(Supplementary table 5-1). There was a significant statistical difference in frequencies between DISH/CC patients and controls (p=0.03) and when adjusted for gender it was statistically different in females (p=0.03), but not in males (Table 5-3). Similar results for significance were obtained using the Cochran-Armitage trend and allelic tests (Table 5-4).

Table 5-4. Statistical results using the Cochran-Armitage trend and allelic tests of genetic variants found in *RSPO4* gene in Azorean patients with DISH/CC and controls without DISH/CC disease.

SNP (A1/A2)	DISH/CC (A1/A2)			Controls (A1/A2)			Test	DISH/CC vs Controls					
								All		M		F	
	All N=110	M N=72	F N=38	All N=72	M N=28	F N=44		CHISQ	P	CHISQ	P	CHISQ	P
rs146447064 (T/C)	5/105	4/68	1/37	10/62	2/26	8/36	T	3.73	0.05	0.10	0.76	3.10	0.08
							A	5.02	**0.03**	0.09	0.76	5.05	**0.02**
rs149154047 (A/G)	5/105	2/70	3/35	7/65	4/24	3/41	T	2.04	0.15	5.06	**0.02**	0.04	0.85
							A	1.89	0.17	4.73	**0.03**	0.03	0.85
rs6056520 (T/C)	31/79	24/48	7/31	14/58	6/22	8/36	T	1.41	0.23	0.95	0.33	0	0.89
							T	1.79	0.18	1.36	0.24	0	0.98
rs150446609 (C/A)	5/105	3/69	2/36	1/71	0/28	1/43	A	0.82	0.36	0.73	0.39	0.31	0.58
							T	1.36	0.24	1.20	0.27	0.52	0.47
rs775644973 (A/G)	1/109	0/72	1/37	0/72	0/28	0/44	A	0.66	0.42	NA	NA	1.19	0.28
							T	0.66	0.42	NA	NA	1.17	0.28
rs6140807 (A/G)	2/108	2/70	0/38	0/72	0/28	0/44	T	1.34	0.25	0.81	0.37	NA	NA
							A	1.32	0.25	0.79	0.37	NA	NA
rs201485021 (T/C)	0/110	0/72	0/38	1/71	0/28	1/43	T	1.55	0.21	NA	NA	0.89	0.35
							A	1.54	0.22	NA	NA	0.87	0.35
rs41275604 (G/C)	4/106	2/70	2/36	2/70	0/28	2/42	T	0.08	0.78	0.81	0.37	0.02	0.90
							T	0.10	0.75	0.79	0.37	0.02	0.88
rs61740632 (C/A)	4/106	4/68	0/38	1/71	0/28	1/43	T	0.85	0.36	1.69	0.16	0.89	0.35
							A	0.82	0.36	1.62	0.20	0.87	0.35

Abbreviations: SNP- Single nucleotide polymorphism**,** M-Males, F-females, T-trend, A- allelic, OR- odds ratio, P- p-value, N- number of individuals, A1- allele 1, A2- allele 2, M- males, F- females, NA- Not applicable.

The other regulatory region variant **rs149154047** in heterozygosity was found in 9% of DISH/CC patients and in 19% of the controls (Supplementary Table 5-1). There was a significant statistical difference in frequencies between DISH/CC male and control males; the frequency of the "G/A" genotype in male DISH/CC was significantly lower than in males of the control group (p=0.05) (Table 5-3). But, significant statistical differences were not found in female DISH/CC patients relative to control females (Table 5-3). Similar results, but with a more significant p-value were obtain using the Cochran-

Armitage trend test (p=0.02, CHISQ=5.06, Df=1) and allelic test (p=0.03, CHISQ=4.73, Df=1) (Table 5-4).

5.4.2 *LEMD3* sequencing

All the coding region of *LEMD3* gene had previously been sequenced in probands from the 4 IBD/IBS families. Three intronic (rs11175678, rs11610822 and rs10534559) and 1 missense mutation (rs201930700) were found (Table 5-5). In this study only the missense variant rs201930700 was investigated.

Table 5-5. Genetic variants identified in *LEMD3* gene in the four probands, previously investigated, and functional significance information.

Exon/Intron	Variant	SNP	Type of variant	MAF	SIFT	PolyPhen	Family/Proband
Intron 1	C.1523 -12 C>T	rs11175678	Intronic	0.04	NA	NA	AZ1/ II:1 (Ht)
Intron 5	c. 1695 +100 G>A	rs11610822	Intronic	0.25	NA	NA	AZ1/ II:1 (Ht)
							AZ4/ III:5 (Ht)
Intron 7	c.2024 -34_2024 -31 del GATT	rs10534559	Intronic (deletion)	?	NA	NA	AZ1/ II:1 (Ht)
							AZ3/II:1 (Hm)
							AZ4/ III:5 (Ht)
Exon 13	c.2701 C>T	rs201930700	Missense	?	0	0.995	AZ3/II:1 (ht)

Abbreviations: SNP- Single nucleotide polymorphism, MAF- Minor allele frequency, SIFT- Sorting Intolerant From Tolerant, PolyPhen- Polymorphism Phenotyping, Ht- heterozygous, Hm- homozygous, NA- not applicable.

Variant rs201930700 was located in exon 13 of the *LEMD3* gene and the SIFT score of 0 and PolyPhen value of 0.995 indicates that this variant has a deleterious and a damaging effect on the protein, respectively. The variant was extremely rare, the MAF was unknown due to insufficient data to establish population frequency, it was identified in only 5 of 121412 alleles (ExAc_Aggregated_Populations), indicating that it was unquestionably very rare (MAF 0.00004). A cohort of 124 individuals, all from Terceira Island, was typed for this mutation and one other individual carrying the variant was identified. This individual and his family were examined (interviewed for clinical purposes, x-rayed and typed). The two families that presented the rs201930700 variant were further investigated (Figure 5-2).

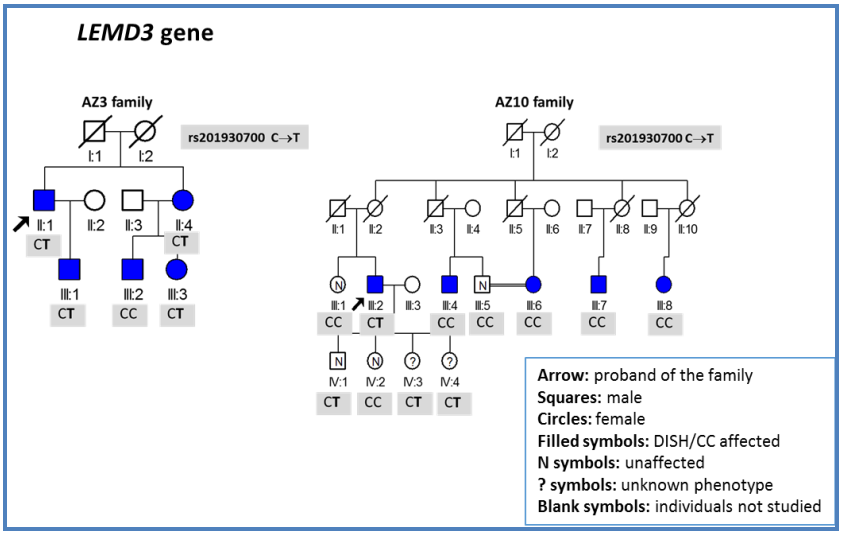


Figure 5-2. Typing results for *LEMD3* gene in DISH/CC families.

The rs201930700 variant was in HWE. Of the 5 individuals studied in the AZ3 family, 4 individuals (2 males and 2 females) were DISH/CC affected and carriers of the rs201930700 variant and 1 individual (male) was DISH/CC affected however, he was a non-carrier of the variant (Figure 5-2). In the AZ3 family it was impossible to verify segregation since all the individuals studied were DISH/CC affected, so the association test was not performed in this family.

Interestingly, the male individual from the AZ10 family found in the randomized population of 124 individuals that were tested for the rs201930700 variant, had DISH/CC disease, as did some of their relatives. Of the 11 individuals studied in the AZ10 family, 5 individuals (3 males and 2 females) were DISH/CC affected, in which one individual was a carrier of the rs201930700 variant and the other 4 were wild type. The variant was also found in 1 male of the 4 unaffected individuals (2 males and 2 females) and in 2 younger females (Figure 5-2). As can be seen in figure 5-2, the majority of individuals in the AZ10 family were DISH/CC affected and apparently no segregation occurs in this family

.

5.5 Discussion

DISH/CC is a poorly understood phenotype characterised by peripheral and axial enthesopathic calcifications, fulfilling the radiological criteria for DISH, and in some cases associated with CC [26, 356, 357]. The concurrence of DISH and CC suggest a shared pathogenic mechanism [24]. The aetiology of DISH/CC is unknown, however, since it is a bone forming disease it is expected that genes related to the calcification and ossification process are implicated in its aetiology. The two genes investigated in the present study, *RSPO4* and *LEMD3*, fulfil the expectations of good candidate genes. In the present study we have found two interesting variants in *RSPO4* gene (rs146447064 and rs14915407) which are significantly more frequent in controls than in DISH/CC patients indicating a possible protective role for these variants in the DISH/CC phenotype. According to what we know the variants rs146447064 and rs14915407 were not associated with Nail disorder or Anonychia (MIM #206800), an autosomal recessive disorder caused by a homozygous or compound heterozygous mutation in the *RSPO4* gene [355]. The rs146447064 variant is a regulatory region variant located in the promoter region, which indicates that this variant could affect the expression of *RSPO4*. It is an extremely rare variant with a MAF value of 0.01 in all populations and 2% in European populations. Interestingly this variant, that in our study gave a protective effect against the disease, does not exist in Asian populations, where the prevalence of OPLL is higher, a disorder very similar to DISH. In addition, the variant is significantly more frequent in control females (p=0.02) than in control males, which could be explained by the fact that 3 control females were homozygous for this rare variant. The variant was also found in heterozygosity in four controls (2 females and 2 males) and in 5 DISH/CC patients (1 female and 4 males).

The rs149154047 variant is also a regulatory region variant located in the promoter region (20: 1002275-1002283). It is also an extremely rare variant with a MAF value of 0.01 in all populations and 3% in European populations. The variant rs149154047 was not associated with the occurrence of the DISH/CC phenotype, however when adjusted for gender, the analysis revealed a significant association between the A allele of the rs149154047 variant and the occurrence of the DISH/CC phenotype in control males (p=0.02) but not in control females. The variant was also found in heterozygosity in 7 controls (3 females and 4 males) and in 5 DISH/CC patients (3 female and 2 males). As far as we known no phenotype was associated with this variant.

The R-spondin proteins activate Wnt/beta-catenin signaling pathways [358] through LRP6 (low density lipoprotein receptor related protein 6) by antagonizing Dickkopf (DKK1) function, which is an inhibitor of osteoblastogenesis and its lower levels are linked to new bone formation [359]. A recent study show, that DISH patients have the levels of total serum DKK1 significantly lower than in healthy controls [360]. It is known that the induction of the Wnt signaling pathway by R-spondin proteins may be a direct consequence of DKK1 inhibition [359, 361]. Based on this knowledge we hypothesise that rs146447064 and rs14915407 are variants associated with the reduction in *RSPO4* gene expression, thus reducing Wnt activation and consequently enhanced the DKK1 which protects against bone formation. However, further studies are needed to ascertain this theory.

In the *LEMD3* gene the missense variant, c.2701 C>T (rs201930700) was found in two families. This variant has been identified very few times previously and both families in which it was typed present the phenotype DISH/CC. This variant causes the substitution of amino acid 901 from a large and basic arginine to a large and aromatic tryptophan. According to the Ensembl database the modified nucleotide is highly conserved in all vertebrates and is located in the carboxyl-terminal nucleoplasmic region of the Man1 protein. This region (amino acids 782-911) is predicted to be an RNA recognition motif-like (RRM-like) protein interaction domain named the U2AF homology motif [362, 363]. This conserved region that contains the UHM domain (U2AF homology motif kinase 1) is exclusive to Man1 proteins and is essential for smad2 and smad3 binding [364]. It is known that interaction between Man1 and Smad1 or Smad2 and Smad3 inhibits bone morphogenic protein (BMP) and TGF-β signaling, respectively [352, 365]. It is reported that heterozygous loss-of-function mutations in *LEMD3* enhance TGF-β signaling leading to sclerosing bone dysplasia osteopoikilosis, and Buschke-Ollendorff syndrome [307]. As this variant (rs201930700) is not a loss-of-function mutation, the carriers of this variant do not present any signs of Osteopoikilosis or Buschke-Ollendorff Syndrome. The effect that the rs201930700 variant produces in the investigated phenotype is difficult to ascertain at this point. We postulate that the rs201930700 variant may lead to enhanced TGF-β signaling, leading to increased bone formation. Our hypothesis was not confirmed by the segregation analysis which might be explained by the characteristics of the sample, in which almost all individuals were DISH/CC affected, making it very difficult to verify segregation. Other studies are necessary to verify the importance of this rare variant on the phenotype under study in order to establish a possible association.

In conclusion, our results suggest a protective role for two *RSPO4* gene regulatory variants, probably by altering gene expression of the *RSPO4* gene. The relevance of the extremely rare variant of *LEMD3* (rs201930700) in the phenotype DISH/CC is difficult to ascertain at this point. To our knowledge, this study is the first to investigate the relationship between *RSPO4* and *LEMD3* genes and DISH and or CC diseases.

5.6. Supplementary material

Supplementary table 5-1. Results of genetic variants found in the *RSPO4* gene in Azorean patients with DISH/CC compared to the controls.

SNP	Genotype	DISH/CC			CONTROLS		
		All: n=55			All: n=36		
		Female: n=19			Female: n=22		
		Male: n=36			Male: n=14		
		All n(%)	Female n (%)	Male n (%)	All n(%)	Female n (%)	Male n (%)
rs146447064	C/C	50 (91)	18 (95)	32 (89)	29 (81)	17 (77)	12 (86)
	C/T	5 (9)	1 (5)	4 (11)	4 (11)	2 (9)	2 (14)
	T/T	0 (0)	0 (0)	0 (0)	3 (8)	3 (14)	0 (0)
rs149154047	G/G	50 (91)	16 (84)	34 (94)	29 (81)	19 (86)	10 (71)
	G/A	5 (9)	3 (15)	2 (6)	7 (19)	3 (14)	4 (29)
	A/A	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
rs6056520	C/C	31 (56)	12 (63)	2 (6)	25 (69)	15 (68)	10 (71)
	C/T	17 (31)	7 (37)	10 (28)	8 (22)	6 (27)	2 (14)
	T/T	7 (13)	0 (0)	7 (19)	3 (8)	1 (5)	2 (14)
rs150446609	A/A	52 (95)	18 (95)	34 (94)	35 (97)	21 (95)	14 (100)
	A/C	1 (2)	0 (0)	1 (3)	1 (3)	1 (4)	0 (0)
	C/C	2 (4)	1 (5)	1 (3)	0 (0)	0 (0)	0 (0)
rs775644973	G/G	54 (98)	18 (95)	0 (0)	36 (100)	22 (100)	14 (100)
	G/A	1 (2)	1 (5)	0 (0)	0 (0)	0 (0)	0 (0)
	A/A	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
rs6140807	G/G	53 (96)	19 (100)	34 (94)	36 (100)	22 (100)	14 (100)
	G/A	2 (4)	0 (0)	2 (6)	0 (0)	0 (0)	0 (0)
	A/A	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
rs201485021	C/C	55 (100)	19 (100)	36 (100)	35 (97)	21 (95)	14 (100)
	C/G	0 (0)	0 (0)	0 (0)	1 (3)	1 (5)	0 (0)
	G/G	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
rs41275604	C/C	53 (96)	18 (95)	34 (94)	34 (94)	20 (91)	14 (100)
	C/T	2 (4)	0 (0)	2 (6)	2 (6)	2 (9)	0 (0)
	T/T	1 (2)	1 (5)	0 (0)	0 (0)	0 (0)	0 (0)
rs61740632	A/A	51 (93)	19 (100)	32 (89)	35 (97)	21 (95)	14 (100)
	A/C	4 (7)	0 (0)	4 (11)	1 (3)	1 (5)	0 (0)
	C/C	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Abbreviations: SNP- Single nucleotide polymorphism, n- number of invividuals, %- percentage, MAF- Minor allele frequency, SIFT- Sorting Intolerant From Tolerant, PolyPhen- Polymorphism Phenotyping, Ht- heterozygous, Hm- homozygous, NA- not applicable.

CHAPTER VI: WHOLE EXOME SEQUENCING

6. WHOLE EXOME SEQUENCING OF PATIENTS SHOWING EXUBERANT ECTOPIC CALCIFICATIONS IN THE AXIAL AND APPENDICULAR SKELETON

6.1. Abstract

Diffuse idiopathic skeletal hyperostosis (DISH) is a common skeletal disorder characterized by the presence of new bone formation in ligaments and entheses. To date, only two susceptibility genes (*COL6A1* and *FGF2*) have a weak genetic link to DISH. In order to identify genetic variants associated with DISH we performed whole exome sequencing in four patients with ectopic calcifications that displayed the rare coexistence of two different rheumatic entities: Diffuse Idiopathic Skeletal Hyperostosis (DISH, MIM 106400) and chondrocalcinosis (CC).

DNA was extracted by a standard salting out procedure. Four DISH/CC patients from different affected families were selected (two females and two males). Sequencing of all coding regions and intron-exon boundaries was performed using an ABI-SOLiD platform. Exome data were filtered in order to find a variant or a group of variants that could be associated with the DISH/CC phenotype. The variants of interest were subsequently confirmed by Sanger sequencing. Selected variants were screened in different pedigrees and in a cohort of DISH/CC patient's vs controls. The statistical analysis was performed using PLINK V1.9.

We successfully identified 21 relevant genetic variants in 17 genes that were directly or indirectly related to mineralization. The commonly used algorithms; SIFT and PolyPhen revealed that several of the gene variants were predicted to be deleterious and damaging to the coded proteins. We identified a significant association between DISH/CC disease and a genetic variant in *BMP4* (rs17563), a gene involved in endochondral bone formation (p=0,009; OR=2.331).

The results of the present study revealed that the variant rs17563 in *BMP4* gene was significantly associated with DISH/CC phenotype. Further studies with an enlarged number of samples will be needed to clarify this association with the phenotype under study.

Keywords: WES, *BMP4*, *ABCC6*, DISH, CC, variants.

6.2 Introduction

Whole exome sequencing is a technique that makes use of the massive parallel sequencing capabilities of next-generation platforms to rapidly identify rare variants in the ~1% of the genome that codes for proteins. The power of exome sequencing comes from the fact that the majority of monogenic diseases arise from mutations within this protein-coding portion of the genome. Furthermore, whole-exome sequencing is now a realistic strategy for detecting pathogenic variants in families in which linkage analysis was not conclusive. Most exome sequencing studies are based on a small number of samples for identification of the causal variants for disease and PCR and Sanger sequencing is then used to extend and reinforce the identified causal variants in a greater number of individuals [366, 367].

Previous studies, undertaken by our group, led to the identification and characterization of twelve families multiply affected with DISH and/or Chondrocalcinosis (CC). A common pathogenic mechanism, was suggested to be shared by the two conditions [24]. Diffuse idiopathic skeletal hyperostosis (DISH, MIM 106400) is a common skeletal disorder characterized by progressive calcification and ossification of ligaments and entheses [1, 2]. The exact prevalence and incidence of DISH is unknown, however it is well known that DISH is more frequent in males and its prevalence rapidly increases with age, affecting mainly subjects over the age of 40 [5]. Weinfeld and colleagues found the prevalence of DISH in patients over 50 years of age to be 25% in males and 15% in females [368], and this disease is becoming a serious problem in aging societies. DISH can co-exist with a great number of other similar rheumatic diseases, and examples of these are the ossification of the posterior lateral ligament (OPLL, MIM 602475) [6], Ossification of the ligamentum flavum [32], Ankylosing spondylitis (MIM 106300) [7-21] and CC [22, 23]. CC is characterized by the deposition of crystals of calcium pyrophosphate (CPP) in articular hyaline and fibro-cartilage [312]. For the moment *ANKH* (CCAL2; #118600) is the only monogenic cause identified for chondrocalcinosis [269], and one study shows that the gene *TNFRSF11B*, encoding osteoprotegerin, is involved in the development of osteoarthritis with chondrocalcinosis [301]. Several lines of evidence suggest that genetic factors may play a part in the aetiology of DISH, such as the existence of familial cases with early onset (in the third decade of life) [64] and the higher frequency of DISH in a specific dog breed, the boxer [77, 78]. So far, however, no single gene has been conclusively associated with the disease. Molecular genetic studies,

therefore, are important to the understanding of the genetic aetiology of DISH. Several linkage and association studies have identified candidate genes/loci that could be linked to DISH susceptibility, including Human Leukocyte Antigens (HLA), Collagen 6A1 gene (*COL6A1*) [34], Fibroblast Growth factor 2 (*FGF2),* [147], Vitamin D (1,25-Dihydroxyvitamin D3) Receptor (*VDR)* and Collagen Type $I_{\alpha 1}$ (*COL1A1*) [145]. However, none of the preceding genes have been demonstrated to be pathogenetically relevant for DISH patients.

DISH is considered a bone forming disease and so genes related to the calcification and ossification process are considered good candidate genes for this disease. In the present study, we performed targeted exome sequencing on four DISH/CC patients, with an apparently autosomal dominant DISH/CC phenotype. The aim was to capture rare and pathogenic variants that are expected to have potentially damaging effects on protein function that leads to modified calcification and/or ossification. To our knowledge this study represents the first report of exome sequencing analysis in DISH disease.

6.3 Material & methods

6.3.1. Subjects

This study involved four patients from four distinct DISH/CC families (AZ1-AZ4) which were selected for whole exome sequencing (2 males and 2 females; age of onset around 40 years). The four families (AZ1-4) contain 35 members (20 males and 15 females; mean age of onset, 36 years; range, 20-50) in which 33 were affected with DISH/CC and 8 had no signs of the disease (see material and methods of this thesis). Standard X rays were taken from: knees, axial skeleton, wrists, hands, elbows, and pelvis. The 4 patients were radiologically characterized (Table 6-1). A group of 55 unrelated Azorean patients with a diagnosis of DISH/CC (36 male, 19 female; age of onset around 40 years) and 36 unrelated healthy controls with a similar ethnic background (16 males and 20 females; mean current age, 68 years; range, 57-102) were also included. This study was approved by the HSEIT Ethics Committee and all participants provided informed consent.

Table 6-1. Radiology results from four selected patients for WES.

Patient	Sex	Radiology					Age at onset	Other diseases
		C-Spine	T-Spine	L-Spine	Knees	Elbow		
AZ1	**M**	Normal	Normal	Sindesmophytosis Inc DISH	?	Enthesopathy ; calcifications	<40	Obesity
AZ2	**F**	DISH inc C5-C6	DISH	DISH	Arthrosis, enthesopathy	Enthesopathy, osteophytosis, calcifications	30	Lithiasis, Diabetes mellitus
AZ3	**F**	N/A	DISH inc	DISH inc	Enthesopathy, osteophytosis, arthrosis	Calcifications, enthesopathy	?	Obesity
AZ4	**M**	Normal	Sindesmophytosis	Syndesmophytosis, anterior osteophytosis	Osteophytosis , calcifications in capsule, arthrosis	Calcifications, enthesopathy osteophytosis	<40	Lithiasis, cardiac arrythmia

Definition of Radiological terminology 1) DISH: continuous ossification along the anterolateral aspect of four contiguous vertebral bodies. **DISH/CC inc:** Continuous ossification along the anterolateral aspect of two or three contiguous vertebral bodies. **2) Sindesmophytosis:** Vertical and symmetrical calcification of the lateral margins of the intervertebral disc space. **3) Osteophytosis:** presence of spurs, which are outgrowths of bone tissue. **4) Enthesopathy:** calcification/ossification process at the site of the insertion of ligaments, tendons, fascia or articular capsule into bone**. 5) Arthrosis:** presence of joint space narrowing, sclerosis and osteophytosis.
Abbreviations: M- male, F- female, DISH- diffuse idiopathic skeletal hyperostosis, C-Spine- Cervical spine, T- Spine- Thoracic spine, L-Spine- Lumbar spine, ?- unknown, N/A- not available.

6.3.2. Exome capture

The selection of patients was made after ruling out mutations in *ANKH*, the only monogenic disease causing gene yet known for CC. All secondary causes for CC were also ruled out by appropriate biochemical testing. DNA was extracted using a standard salting out procedure. Samples were resequenced, using an Applied Byosystems - Sequencing by Oligonucleotide Ligation and Detection platform (ABI-SOLiD) and Agilent's SureSelect Target Enrichment System for 38 Mb, by "Sistemas Genómicos, S.L." in Valencia, Spain. The quality and quantity of extracted DNA was evaluated by agarose gel electrophoresis, measurement of absorbance at 260 nm was established using a NanoDrop 1000 and Qubit fluorescence quantification. SOLiD Fragment libraries were prepared and enriched with SureSelect All Human Enrichment Target Exon. The quality and quantity of the libraries were assessed by analysis with Agilent 2100 Bioanalyzer and Qubit. Each library went through a process of emulsion PCR for clonal amplification of the fragments, followed by an enrichment process and chemical modification to allow loading in the reaction chamber. The quality and quantity of the beads obtained for each library were estimated taking into account the parameters given by Work Flow Analysis. Then, ligation sequencing was done to obtain sequences of 50 nucleotides +35nucleotides

(Paired-end) in SOLiD4. The data quality was estimated using the parameters provided by the software SETS (SOLiD Experimental Tracking System). Single Nucleotide Variants (SNVs) were classified using Ensembl's nomenclature and grouped using the following the scheme: Known and Novel (Coding, Splicing, Others). The coding variants were divided into Non-synonymous and Synonymous. The `Others´ included intronic, untranslated (UTR), regulatory region, intergenic, downstream and upstream variants.

6.3.3. WES filtering

Three filtering strategies were applied in order to find a variant or a group of variants that could be associated with the DISH/CC disease.

6.3.3.1. Filtering candidate genes provided by WES results

Two different models (dominant and recessive) were used to identify candidate genes. The most probable model for inheritance of the disorder under study is an autosomal dominant model. Taking into consideration that the majority of the families used for the study come from a restricted area of the Terceira Island, a genetic founder effect was expected. A recessive model cannot be ruled out, due to the high consanguinity present on the island, but it is less likely due to the distribution pattern of the disease in pedigrees.

A list of 815, 917, 872 and 593 genes, was generated to include the candidate genes under a dominant model (Table 6-2). These genes were then filtered based on sharing between the four investigated DISH/CC patients. A group of 52 genes were common to the four patients (Table 6-3), and from this group the candidate genes for testing were selected taking into consideration their function. For this reason priority was given to candidate genes involved in the calcification and/or ossification process or related conditions that could be associated with DISH/CC disease.

Table 6-2. Number of candidate genes per sample.

Samples	Number of candidate genes	
	Dominant model	Recessive Model
AZ1	815	48
AZ2	917	58
AZ3	872	47
AZ4	593	25

There was only one gene that came out as a good candidate gene for a recessive model: HLA-*DQA2* (Table 6-3), which was also present in the list of candidate genes when using

a dominant model of inheritance. There is not much information available about the *HLA-DQA2* gene and the IPD-IMGT/HLA Database does not have typing results for this gene. The study of this gene was not therefore carried out in the present study, due to time constraints.

Table 6-3. Number of candidate genes shared by the investigated DISH/CC patients.

Patients	Number of candidate genes shared:	
	Dominant model	Recessive Model
AZ1+AZ2+AZ3+AZ4	**52**	**1**
AZ1+AZ2+AZ3	118	2
AZ1+AZ2+AZ4	65	1
AZ2+AZ3+AZ4	78	2
AZ1+AZ3+AZ4	77	1
AZ1+AZ2	220	6
AZ1+AZ3	212	4
AZ1+AZ4	139	4
AZ2+AZ3	249	6
AZ2+AZ4	149	4
AZ3+AZ4	162	3

We then procured all the variants in the shared genes and focused on nonsynonymous, splice sites, stop loss/gain and frameshift variants, anticipating that synonymous and intronic variants would be far less likely than functional variants to be relevant in the pathogenicity.

6.3.3.2. Filtering within candidate genes

A group of 20 candidate genes was selected through a literature search, and included genes that are possibly involved in bone metabolism and/or related conditions. These genes are Alkaline Phosphatase (*ALPL/TNAP*), the Calcium Sensing Receptor (*CASR*), Bone morphogenetic protein receptor type 1B (*BMPR1B*), Osteopontin (*OPN/SPP1*), Integrin binding sialoprotein (*IBSP*), Fibroblast Growth factor 2 (*FGF2*), Inorganic Pyrophosphate Transport Regulator (*ANKH*), Collagen type XI, alpha 2 (*COL11A2*), Nucleotide pyrophosphatase 1 (*ENPP1*), Runt-related transcription factor (*RUNX2*), Dickkopf WNT signaling pathway (*DKK-1*), Insulin like growth factor 1 (*IGF1*), Matrix Gla protein (*MGP*); Vitamin D (*VDR*), Bone morphogenetic protein 4 (*BMP4*), Collagen type 1 alpha 1 (*COL1A1*), Transforming growth factor beta 1 (*TGFβ*1), Solute carrier family 29 member 1 (*SLC29A1*), Bone morphogenetic protein 2 (*BMP2*) and Collagen type VI, alpha 1 (*COL6A1*). The genes selected were screened against the WES results

and the variants were annotated in an excel file. Subsequently we focused only on nonsynonymous, splice sites, loss or gain of a stop and frameshift variants.

6.3.3.3. Filtering pathogenic variants

In this filtering strategy we used two levels; variant and knowledge level (Figure 6-1). In the variant level we first filtered according to variant type and focused on variants with functional significance (splice sites, frameshift coding, nonsynonymous coding, lost or gained stop variants. Only the SIFT prediction with Damaging and Unknown and GERP values equal or higher than 3 were included. We excluded variants with MAF values higher than 0.05 by filtering the SNP_IDs (rs number or by chromosome position) against a public Ensembl tool, Variant Effect Predictor (http://www.ensembl.org/info/docs/tools/vep/index.html). Then at the "knowledge level" we focused only on genes associated with calcification and/or ossification processes or related conditions. Lastly we evaluated the functional significance and pathogenic potential of each variant found in the selected genes.

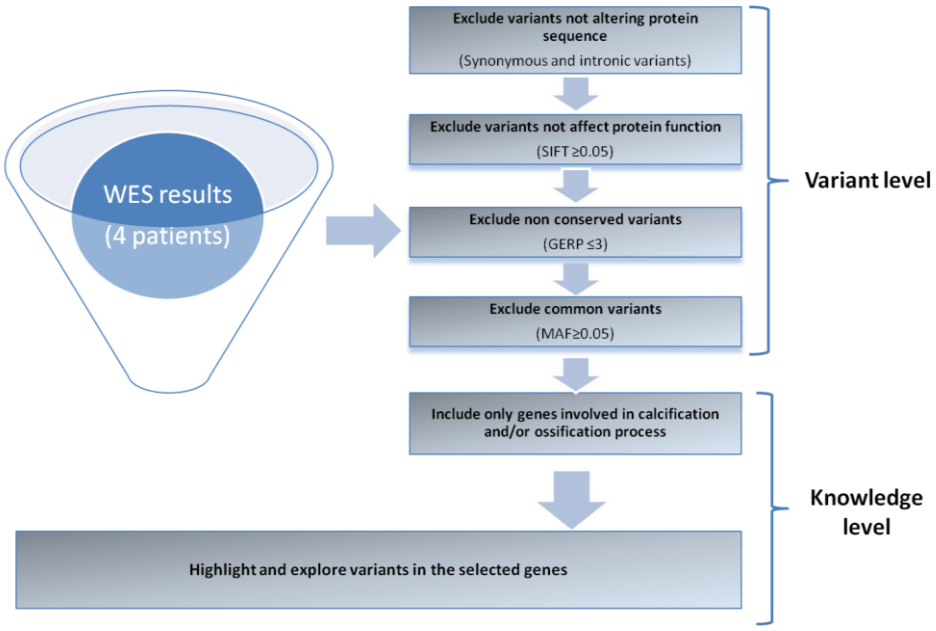


Figure 6-1. The two-level filtration approach used to analyze the WES results from 4 patients with DISH/CC disease. WES: Whole exome sequencing, SIFT: Sorting Intolerant From Tolerant, GERP: Genomic Evolutionary Rate Profiling score and MAF: Minor Allele Frequency.

6.3.4. Validation and Evaluation of Genes/Variants

PCR primers were designed using the software Primer3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) to amplify and validate variants detected by Exome Sequencing (primers sequences and PCR conditions are indicated in the materials and methods of this thesis). PCR products were purified using ExoSAP-IT™ following the manufacturer's instructions. After purification with ExoSAP-IT, sequencing reactions with BigDye® Terminator were performed unidirectionally, or when necessary, bidirectionally. Sequencing reactions were purified with acetic acid and EDTA followed by ethanol precipitation. The templates were ressuspended in Hi-Di formamide and analysed using an automated DNA sequencer ABI 3130xl (Applied Biosystems®). Genetic variants were screened using Sequencing analysis and SeqScape software's (Applied Biosystems®) and using as a reference NCBI sequences (see the material and methods section of this thesis for more detail).

The information about each gene was obtained from several databases, including Ensembl (http://www.ensembl.org/index), National Center for Biotechnology Information (NCBI) (http://www.ncbi.nih.gov) and Pubmed (https://www.ncbi.nlm.nih.gov/pubmedwe), GeneCards (http://www.genecards.org/), Online Mendelian Inheritance in Man (OMIM) (http://www.omim.org/) and MalaCards (http://www.malacards.org/).

The functional significance and the potential deleterious effect of each variant was explored in the following databases: Ensembl (http://www.ensembl.org/index), Human Gene Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk/ac/index.php) and dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP/), making use of algorithms, such as PolyPhen-2 (Polymorphism Phenotyping v2) (http://genetics.bwh.harvard.edu/pph2/) that defines severity as, benign, [0-0.2], possibly damaging (0.2-0.85), and probably damaging [0.85-1]) and for SIFT, damaging if less than 0.05 and for GERP, this ranges from -12.3 to 6.17, with 6.17 being the most conserved [369]). The MAF values of each variant were also analyzed. Protein conservation analysis was performed using ClustalW (http://www.genome.jp/tools/clustalw/) to compare homologous amino acid sequences among multiple vertebrates at the sites where the variations occur. The accession numbers of the transcript are available in supplementary table 6-1.

6.3.5. Association studies

In order to verify a possible association with DISH/CC phenotype selected variants were screened in family members (affected with DISH/CC and unaffected), and the more conserved variants, those that were present in three or four WES patients, were selected for screening in a group of DISH/CC patients and controls.

6.3.6. Statistical analysis

All SNPs were checked to assess their compliance with the Hardy-Weinberg equilibrium (HWE). For all DISH/CC families, a basic family based association test (transmission disequilibrium test -TDT) was used to assess the allele transmission. The TDT test was calculated for CHISQ, odds ratios (OR) (95% confidence interval) and corresponding p-values. To assess the difference in allele frequencies between the 55 patients with DISH/CC and the 36 control individuals a Fisher exact test was used. The Fisher exact test was calculated for odds ratios (OR) (95% confidence interval) and corresponding p-values. For all statistical tests used a p-value of ≤ 0.01 was considered statistically significant. All statistical analysis were performed using PLINK software [354].

6.4 Results

6.4.1. Exome capture - variants detected

An average of approximately 3.8 billion bases of sequence per patient were generated and the capture specificity and sensibility in all samples was about 55% and 94%, respectively. The average sensibility for all four samples was over 94%. Table 6-4 summarizes the results after the SNV calling and indel identification steps for each sample.

Table 6-4. Known, novel and total number of SNVs and Indels for the four samples analysed (AZ1-4).

Sample	SNVs			Indels			Total	TOTAL/sample
	Known	Novel	Total	Known	Novel	Complex		
AZ1	17281	1619	18900	457	405	51	913	19813
AZ2	18086	1759	19845	440	496	66	1002	20847
AZ3	18327	1650	19977	524	505	73	1102	21079
AZ4	17575	1223	18798	475	424	72	971	19769

Abbreviations: SNVs: Single nucleotide variants

6.4.2. Filtering Results

From the three filtering strategies deployed we obtained 21 missense, deletion and splice site variants in 17 genes: *PLCG2*, obtained in the first filtering strategy that was based on candidate genes in a dominant model; *ALPL, CASR, FGF2, COL11A2, ENPP1, MGP, VDR, BMP4, COL1A1, TGFβ1, BMP2* and *COL6A1*, were obtained from the second filtering based on candidate genes and *FLNC, AMER3, PPP2R2D* and *ABCC6*, were obtained from the third filter based on predicted pathogenic variants (Table 6-5).

After analysis of the functional significance of the variants we found several with a possible effect on protein function and/or low MAF values, which are indicative of a deleterious variant. The variants **c.**2054+7G>A and c.3786G>C (K1262N) in ***PLCG2*** gene are both extremely rare. The c.2054+7G>A is a splice site variation located seven bases after exon 19 and the c.3786G>C (K1262N) is a missense variant which causes substitution of lysine with asparagine at amino acid 1262. The amino acid lysine is basic and polar and asparagine is neutral and polar. The logarithm SIFT (0) in c.3786G>C (K1262N) suggests the variation has a deleterious effect on the PLCG2 protein. The splice site variant c.2054+7G>A was present in patients AZ3 and AZ4 and the missense variant c.3786G>C (N1260K) was present in AZ2 and all were heterozygous (Table 6-5).

Table 6-5. List of variants found by WES and confirmed by Sanger Sequencing.

Gene	Gene function	Chr	SNP	Variant	AA	MAF	SIFT	PolyPhen	Patient			
									AZ1	AZ2	AZ3	AZ4
PLCG2	A calcium dependent phosphatidylinositol-specific phospholipase C, which is crucial in transmembrane signalling. Involved in the Wnt receptor signaling pathway. This gene regulates osteoclastogenesis, and in a mice model, a deletion of this gene leads to an osteopetrotic phenotype [370].	16	rs138158454	c.2054+7G>A	NA	0,01	NA	NA			ht	ht
			rs374430619	c.3786G>C	K1262N	?	0	0,008		ht		
ALPL	Play a role in bone mineralization. Hypophosphatasia in adult (#146300), Infantil (#241500) and Childhood (#241510) are caused by mutations in this gene.	1	rs149344982	c.455G>A	R152H	0,01	0,56	0,077		ht		
			rs3200254	c.787T>C	Y263H	0,27	1	0	ht		ht	
CASR	Plays a pivotal role in systemic calcium metabolism. Loss of function mutations cause hyperparathyroidism neonatal (#239200) [371], whereas gain of function result in hypocalcemia with Bartter syndrome (#601198) [371].	3	rs1801725	c.2956G>T	A986S	0,09	0,22	0,01		ht		ht
			rs1801726	c.3031G>C	E1021Q	0,08	1	0		hm	hm	hm
FGF2	Osteoblast gene expression and differentiation [148]. Diseases associated with FGF2 include Corneal Neovascularization and Crouzon Syndrome. One study associated this gene with DISH susceptibility [147].	4	rs1048201	c.*757C>T	NA	?	NA	NA	ht	ht		ht
COL11A2	Involvement in the ossification process. Mutations in this gene cause Otospondylomegaepiphyseal dysplasia (#215150), Weissenbacher-Zweymuller syndrome (#277610), Fibrochondrogenesis 2 (#614524), Stickler syndrome, type III (#184840) and Deafness (#601868 and #609706). Mutations in this gene may also be associated with OPLL disease (%602475).	6	rs9277934	c.826G>A	E276K	0,32	0.45	0.557	hm	ht	hm	ht
			rs2229792	c.5165C>T	P1722L	0,01	0.01	0.088				ht
ENPP1	Regulates soft-tissue calcification and bone mineralization by producing PPi [89]. Mutations in this gene are associated with Cole disease (#615522), Hypophosphatemic Rickets (#613312) and Arterial calcification generalized of infancy 1(#208000). Mutations in this gene have been also associated with OPLL [165].	6	rs1044498	c.517A>C	K173Q	0,34	0,19	0,014	ht	ht	ht	
MGP	Physiological inhibitor of ectopic calcification. Diseases associated with this gene include Keutel Syndrome (#245150) and Vitamin K deficiency Hemorrhagic Disease.	12	rs4236	c.304A>G	T102A	0,39	1	0	ht		ht	ht
VDR	Involved in mineral metabolism; control the absorption of calcium and phosphate. Diseases associated with this gene include Rickets, vitamin D-resistant (#277440) and osteoporosis (#166710).	12	rs2228570	c.2T>C	M1T	0.35	0	0.995	hm	hm	ht	ht
BMP4	Involved in endochondral bone formation. Diseases associated with BMP4 include Microphthalmia Syndromic 6 (#607932) and Orafacial Cleft 11 (#600625).	14	rs17563	c.455T>C	V152A	0,37	0,57	0,005	hm		hm	ht
COL1A1	Involved in bone maturation, development and mineralization [372]. Mutations in this gene cause Caffey Disease (#114000) and Osteogenesis Imperfecta Type I-IV (#166200, #166210, #259420 and #166220, respectively).	17	rs372029024	c.3247G>T	A1083T	?	0.08	0	hm			
TGFβ1	Bone remodeling; potent stimulator of osteoblastic bone formation. Diseases associated with this gene include Camurati-Engelmann Disease (#131300) and Cystic Fibrosis (#219700).	19	rs55659002	c.713-8delC	NA	?	NA	NA			ht	
BMP2	Induce bone and cartilage formation. Diseases associated are Brachydactyly, Type A2 (#112600) and Hemochromatosis (#235200).	20	rs235768	c.570A>T	R190S	0,24	0	0,977	ht		ht	ht
COL6A1	Play a role in maintaining the integrity of various tissues. Mutations in this gene could cause ectopic bone formation in OPLL and DISH [34, 146].	21	rs1053312	c.2549G>A	R850H	0.27	0,11	0,018		hm		
FLNC	Involved in reshaping of the cytoskeleton. Mutations in this gene cause Myofibrillar myopathy-5 (MFM5 #601419) [373] and distal myopathy-4 (MPD4; MIM 614065), which shows a different pattern of muscle involvement [374].	7	rs2291569	c.4700G>A	R1567Q	0.06	0,01	0.999		ht		
AMER3	Is a positive regulator of Wnt-β-catenin signaling pathway.	2	rs72854996	c.1300C>G	L434V	0.05	1	0	ht			
PPP2R2D	Catalyze the removal of phosphate groups from serine and/or threonine residues by the hydrolysis of phosphoric acid monoesters. The protein belongs to the TGF-β pathway.	10	rs34473884	c.1072G>A	G358S	0,18	0.03	0.173	hm	ht	ht	ht
ABCC6	Unknown function however during the last few years has been extensively related to ectopic calcification. Cause Pseudoxanthoma elasticum (PXE; MIM#264800), a disorder with calcification of the elastic fibres and in some cases can cause arterial calcification generalized of infancy type 2 (GACI2; MIM#614473).	16	rs41278174	c.3190C>T	R1064W	0.01	0	0.932			ht	

Abbreviations: Chr- chromosome, SNP-Single nucleotide polymorphism, AA- Amino acid, MAF- Minor allele frequency, SIFT- Sorting Intolerant From Tolerant, PolyPhen- Polymorphism Phenotyping v2, ht-heterozygous, hm-homozygous, NA- Not Applicable, *PLCG2*- Phospholipase C Gamma 2, *ALPL*- Alkaline Phosphatase, Liver/Bone/Kidney, *CASR*- calcium-sensing receptor, *FGF2*- Fibroblast growth factor 2, *COL11A2*- Collagen Type XI Alpha 2 Chain, *ENPP1*– ectonucleotide pyrophosphatase/phosphodiesterase 1, MGP- Matrix Gla Protein, VDR- Vitamin D Receptor, *BMP4*- Bone morphogenetic protein 4, *COL1A1*- Collagen Type I Alpha 1 Chain, *TGFβ1*- Transforming Growth factor Beta 1, *BMP2*- Bone morphogenetic protein 2, *COL6A1*- Collagen Type VI Alpha 1 Chain, *FLNC*- Filamin C, *AMER3*- APC Membrane Recruitment Protein 3, *PPP2R2D*- Protein Phosphatase 2 Regulatory Subunit B delta, *ABCC6* – ATP-binding cassete subfamily C, member 6.

COL11A2 gene variants c.826G>A (E276K) and c.5165C>T (P1722L) are both missense; the c.826G>A (E276K) causes a glutamate substitution for a lysine at amino acid position 276. The amino acid glutamate is acidic and lysine is basic. The c.5165C>T (P1722L) variant is extremely rare (MAF of 0.01) and causes a proline substitution for a leucine in 1722 protein position. Both amino acids are non-polar. The logarithm PolyPhen (0.557; possibly damaging) in c.826G>A (E276K) and the SIFT (0.01; deleterious) in c.5165C>T (P1722L) suggests a damaging and deleterious effect, respectively on the COL11A2 protein. The c.826G>A (E276K) was identified in homozygosity in AZ1 and AZ3; and in heterozygosity in AZ2 and AZ4. The c.5165C>T (P1722L) was identified in heterozygosity in AZ4 patient (Table 6-5).

The variant c.2T>C (M1T) in the ***VDR*** gene is a missense mutation which causes a methionine substitution for a threonine in amino acid 1 of the protein. The amino acid change is from non-polar (methionine) to polar (threonine). According to the conservation analysis this amino acid position is totally conserved between all mammals analyzed (Supplementary figure 6-1), and the logarithms SIFT (0; deleterious) and PolyPhen (0.995; probably damaging) suggest a strong effect on the VDR protein. The variant was homozygous in AZ1 and AZ2; and heterozygous in AZ3 and AZ4 (Table 6-5).

The variant c.570A>T (R190S) in the *BMP2* gene is a missense which causes an arginine substitution for a serine in amino acid 190 of the protein. The amino acid change is from basic (arginine) to polar (serine). The amino acid position is totally conserved between all the vertebrates studied so far (Supplementary figure 6-1). In addition, the logarithms SIFT (0; deleterious) and PolyPhen (0.977; probably damaging) suggest a strong effect on the BMP2 protein. The variant was found in AZ1, AZ3 and AZ4 in heterozygosity (Table 6-5).

In the ***FLNC*** gene we found the missense variant c.4700G>A (R1567Q) which causes an arginine substitution for a glutamine at amino acid 1567 of the protein. The amino acid change is from a large, basic amino acid (arginine) to a medium and polar amino acid (glutamine). According to the conservation analysis this amino acid position is highly conserved in vertebrates (Supplementary figure 6-1), furthermore the logarithms SIFT score (0.01; deleterious) and PolyPhen (0.999; probably damaging) indicates that this variant has a damaging effect on the protein. The frequency of this variant is relatively low in Europe with a MAF of 0.06. The variant was heterozygous in AZ2 (Table 6-5).

The variant c.1072G>A (G327S) found in ***PPP2R2D*** gene is a missense variant which causes substitution of glycine for serine at amino acid 327 in the protein. These two amino acids are hydrophilic but glycine is non-polar and serine is polar. The amino acid position is totally

conserved in all vertebrates studied (Supplementary figure 6-1), and the variant has a low, deleterious, SIFT score (0.03) but the Polyphen score (0,173) does not corroborate its harmful effect. The frequency of this variant is high in Europe with a MAF of 0.18 and this variant was identified in all four DISH/CC patients used for WES; AZ1 was homozygous and AZ2, 3 and 4 were heterozygous (Table 6-5). The variant c.3190C>T (R1064W) in the ***ABCC6*** gene is a conserved missense variant that seems to be of fundamental importance since the algorithms SIFT score (0; deleterious) and PolyPhen (0.932; probably damaging) indicates that this mutation has a deleterious effect on the protein and is a rare variant (MAF of 0.01). The variant was heterozygous in AZ3 (Table 6-5). We found eight genetic variants, in heterozygous and/or homozygous states, in conserved positions in proteins associated with mineralization; four variants were in regions that are normally highly conserved across the vertebrates (*CASR* ((rs1801725), *BMP2* (rs235768), *FLNC* (rs2291569) and *PPP2R2D* (rs34473884)), and four other variants were in positions normally conserved in mammals (*VDR* (rs2228570), *BMP4* (rs17563), *COL1A1* (rs372029024) and *ABCC6* (rs41278174)) (Table 6-6).

Table 6-6. Conservation analysis of the variants identified in this study. In bold are represented the conserved variants found in at least 3 patients.

Patient (sex)	Mutational spectrum		
	Highly conserved	**Conserved between mammals**	**Relaxed regions**
AZ1 (M)	*BMP2* (rs235768/ht) *PPP2R2D* (rs34473884/hm)	*VDR* (rs2228570/hm) *BMP4* (rs17563/hm) *COL1A1* (rs372029024/hm)	*ALPL* (rs3200254), *ENPP1* (rs1044498), *MGP* (rs4236), *AMER3* (rs72854996), *COL11A2* (rs9277934).
AZ2 (F)	*CASR* (rs1801725/ht) *FLNC* (rs2291569/ht) *PPP2R2D* (rs34473884/ht)	*VDR* (rs2228570/hm)	*CASR* (rs1801726), *COL6A1* (rs1053312), *PLCG2* (rs374430619), *ALPL* (rs149344982), *COL11A2* (rs9277934), *ENPP1* (rs1044498).
AZ3 (F)	*BMP2* (rs235768/ht), *PPP2R2D* (rs34473884/ht)	*BMP4* (rs17563/hm) *ABCC6* (rs41278174/ht)	*PLCG2* (rs138158454), *ALPL* (rs3200254), *ENPP1* (rs1044498), *MGP* (rs4236), *VDR* (rs2228570), *TGFB* (rs55659002), *CASR* (rs1801726), *COL11A2* (rs9277934).
AZ4 (M)	*CASR* (rs1801725/ht) *BMP2* (rs235768/ht), *PPP2R2D* (rs34473884/ht)	*VDR* (rs2228570/ht) *BMP4* (rs17563/ht)	*PLCG2* (rs138158454), *COL11A2* (rs2229792), *MGP* (rs4236), *CASR* (rs1801726).

Abbreviations: M- male, F-female, ht-heterozygous, hm-homozygous**,** *PLCG2*- Phospholipase C Gamma 2, *ALPL*- Alkaline Phosphatase, Liver/Bone/Kidney, *CASR*- calcium-sensing receptor, *COL11A2*- Collagen Type XI Alpha 2 Chain, *ENPP1*– ectonucleotide pyrophosphatase/phosphodiesterase 1, MGP- Matrix Gla Protein, VDR- Vitamin D Receptor, *BMP4*- Bone morphogenetic protein 4, *COL1A1*- Collagen Type I Alpha 1 Chain, *BMP2*- Bone morphogenetic protein 2, *COL6A1*- Collagen Type VI Alpha 1 Chain, *FLNC*- Filamin C, *AMER3*- APC Membrane Recruitment Protein 3, *PPP2R2D*- Protein Phosphatase 2 Regulatory Subunit B delta, *ABCC6* – ATP-binding cassete subfamily C, member 6.

As can be seen in table 6-6, all patients have at least 2 highly conserved genetic variants in combination with other variants that are normally conserved in mammals.

6.4.3. Association between variants and DISH/CC phenotype

6.4.3.1. Segregation of variants

We selected 6 variants in genes *PLCG2 (*c.2054+7G>A and c.3786G>C (K1260N)), *FLNC (*c.4700G>A (R1567Q)), *AMER3 (*c.1300C>G (L434V)), *PPP2R2D* (c.1072G>A (G327S)) and *ABCC6* (c.3190C>T/R1064W) to verify the segregation within families of the investigated patients (Figure 6-2).

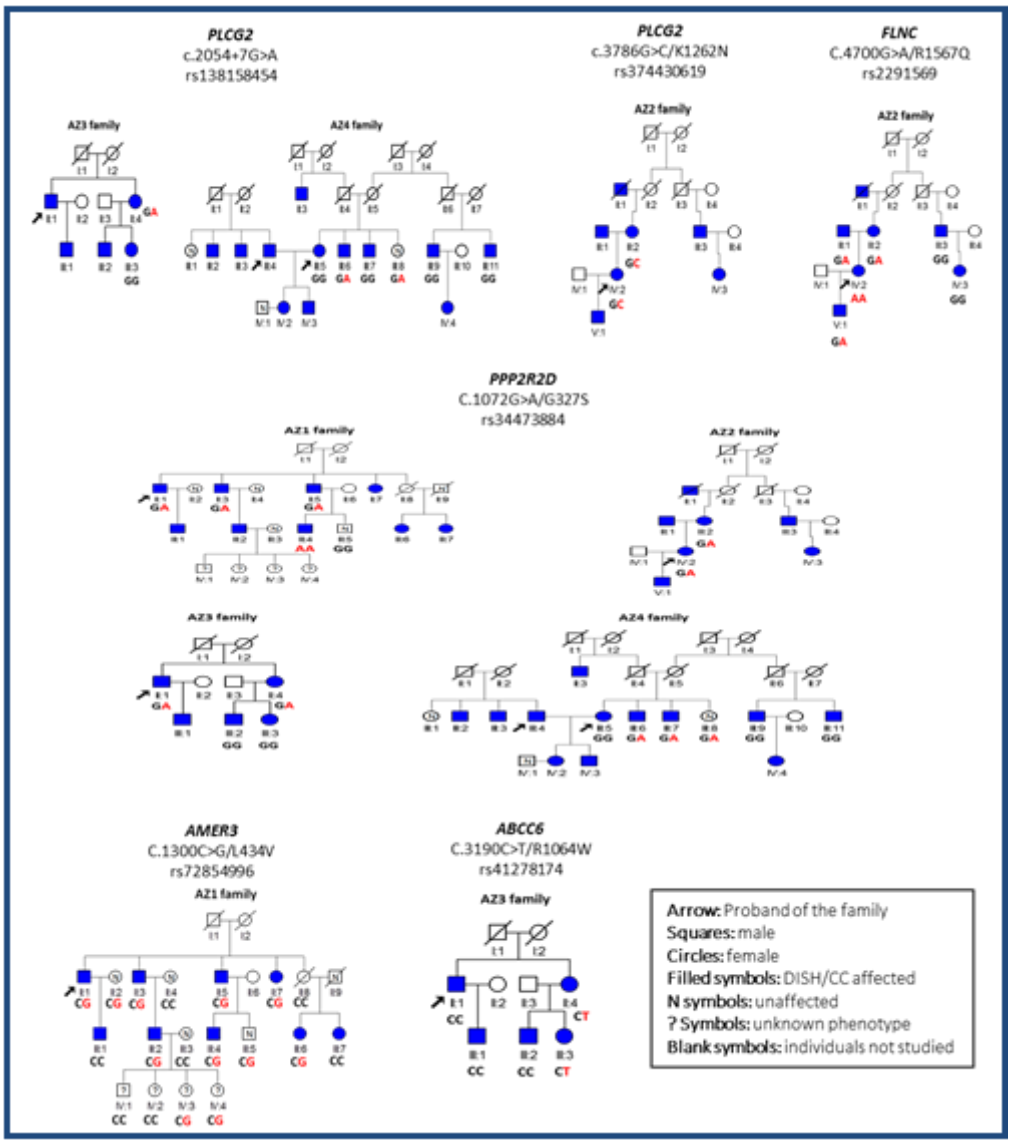


Figure 6-2. Segregation results with DISH/CC phenotype in families AZ1-4. WES sequencing patients: III:4 (AZ1), III-2 (AZ2), II:4 (AZ3) and III:6 (AZ4).

Most of the individuals were affected by DISH/CC disease and this made it difficult to verify segregation in these families. The selected variants, individually studied, did not segregate within families mainly because these families were uninformative and lacked unaffected individuals, and therefore the segregation analysis was not performed for all the candidate genes presented in table 6-5. Although not statistically significant, it was possible to evaluate the variants in *AMER3* and *FLNC* genes (Table 6-7).

Table 6-7. Family based association test (TDT test) results for variants of *AMER3* gene in AZ1 family and *FLNC* gene in AZ2 family.

Gene	SNP	Alleles M/m	TDT			
			TR:UT	OR	CHISQ	P
AMER3	rs72854996	C/**G**	1:2	0.5	0.3333	0.5637
FLNC	rs2291569	G/**A**	2:0	NA	2	0.1573

Abbreviations: SNP- Single nucleotide polymorphism, M/m – major allele/minor allele, TDT- Transmission disequilibrium test, TR:NT-Transmitted/Untransmitted minor allele account, OR- odds ratio, P- pvalue, NA- not applicable.

6.4.3.2. Case/control studies

The variants rs34473884, rs9277934 and rs2228570 in *PPP2R2D*, *COL11A2* and *VDR* genes, respectively, were present in all 4 WES patients. The variants rs1048201, rs235768, rs1044498 and rs17563 in *FGF2*, *BMP2*, *ENPP1* and *BMP4* genes, respectively were present in three of the four WES patients. All these variants had a high degree of conservation. The variants rs4236 in *MGP* gene and rs1801726 in *CASR* gene were present in three of the four WES patients however they were not conserved and therefore the case/control study of these genes was not performed. Seven variants were screened in a group of 55 DISH/CC patients and 36 controls and the results are indicated in table 6-8. The variant rs17563 in *BMP4* gene was found to be more frequent in DISH/CC group than in controls (p=0.009; OR=2.331) (Table 6.8).

Table 6-8. Association study between seven variants from seven genes and DISH/CC phenotype. The risk allele are in bold.

Chr	Gene	SNP	Allele M/m	MAF		OR	p-value
				DISH/CC (N=55)	Controls (N=36)		
4	*FGF2*	rs1048201	C/**T**	0,155	0,139	1,133	0,771
6	*COL11A2*	rs9277934	G/**A**	0,373	0,389	0,934	0,826
6	*ENPP1*	rs1044498	A/**C**	0,191	0,208	0,897	0,773
10	*PPP2R2D*	rs34473884	G/**A**	0,255	0,181	1,550	0,243
12	*VDR*	rs2228570	T/**C**	0,382	0,389	0,971	0,924
14	*BMP4*	rs17563	T/**C**	0,473	0,278	2,331	0,009
20	*BMP2*	rs235768	A/**T**	0,264	0,250	1,074	0,837

Abbreviations: SNP- Single nucleotide polymorphism, M/m – major allele/minor allele, MAF- Minor allele frequency, OR- odds ratio, Chr- chromosome.

6.5. Discussion

Many human diseases appear to have a strong hereditary component. A large number of studies, using the WES technique, have reported variants and genes responsible for several monogenic diseases of unknown causes [163, 366, 375]. In this study, we used WES as a method to identify candidate genes for DISH/CC aetiology, and association studies to investigate specific variants. As expected, thousands of protein coding variants per patient were identified across each exome, which meant a huge number of variants had to be filtered and identification of the causative variant is like "finding a needle in a haystack". Consequently, different filtering strategies were used to find potential high risk variants in genes that could be associated with the DISH/CC phenotype. It is not easy to draw conclusions from segregation study since most of the individuals studied were DISH/CC affected making these families uninformative, however the association study performed in this study indicated that the SNP rs17563 in *BMP4* gene was significantly associated with the DISH/CC phenotype. Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor beta (TGFβ) superfamily. *BMP4* has the same origin as *BMP2* and they are similar in structure and function. *BMP2* and *BMP4* are genes mapping to chromosomes 20 and 14, respectively that encode important regulators of bone formation [376]. In one study, Ono et al [377] found that *BMP2* and *TGF-β* played an important role in ossification in the conditions OPLL, a disorder that is very similar to, and can coexist with DISH [6], and ligamentum flavum, by acting on the progenitor cells in the ligament, causing them to proliferate, form cartilage and then ossify. *BMP4* also seems to

play a crucial role in the pathogenesis of OPLL, as demonstrated in a large scale study by Furushima and collaborators that found evidence of linkage with this gene [159]. Despite the association of *BMP2* and *BMP4* with ectopic bone formation, their association with OPLL is controversial, and there is both evidence in favor [160, 161] and against this idea [72]. Interestingly, Kan *et al* [378] produced a mice overexpressing BMP4 and observed the development of progressive postnatal heterotopic endochondral ossification, a phenotype similar to human Fibrodysplasia ossificans progressive (FOP). In rabbits, recombinant human BMP4 enhances posterior spinal fusion [379]. In the present study we identified two variants in *BMP* genes. The variant (R190S) in the *BMP2* gene had a low SIFT score and a higher, PolyPhen, which predicted a deleterious and a damaging effect on the protein and this variant has previously been associated with the occurrence of OPLL [158] and with Immunoglobulin A nephropathy [380]**.** The missense variant c.455T>C (V152A) in the *BMP4* gene, which in this study was significantly more frequent in the DISH/CC group relative to the control group, was located in the translated region of *BMP4* exon 5 and caused the substitution of a valine by an alanine at amino acid position 152, a position conserved between species (Supplementary figure 6-1). The structure and function of the protein was apparently not significantly affected by the substitution due to physicochemical similarities of the involved amino acids. Furthermore, **t**he algorithms SIFT (0.57; tolerated) and PolyPhen (0.005; benign) did not suggest a strong effect on the protein. However, according to Capasso et al [381] the variant rs17563 affected *BMP4* gene expression; this variant promotes a change in the structure of the mRNA, and the levels of BMP4 mRNA were significantly higher in carriers of this variant relative to non-carriers. Tanno et al [382] found that the mRNA and expression of BMP4 protein were significantly increased in OPLL cells derived from ossified spinal ligament when compared to non OPLL cells. This variant was associated with the occurrence and severity of OPLL in Chinese males [160] and has been reported to affect hip bone density in postmenopausal women [383].

Polymorphisms in other genes such as *COL6A1* [33, 146] and *FGF2* [147] have been r associated with DISH susceptibility, however these associations do not fully explain the disease. The *COL6A1* gene encodes an extracellular matrix protein that might serve as a scaffold for osteoblast or pre-osteoblast cells or chondrocytes that subsequently proceed to membranous or endochondral ossification [34]. According to several authors, this gene is strongly associated with OPLL and certain polymorphisms are considered helpful markers of OPLL [33, 146]. One of these polymorphisms, intron 32 (-29), associated with OPLL was also significantly associated with DISH Japanese patients but not with DISH Czech

patients[34]. In our study, we identified the variant rs1053312 in *COL6A1* and it was homozygous in one WES patient, however no differences were found between DISH/CC patients and controls groups. This variant was already studied in OPLL disease but, as in our study, no association was found [146]. The other gene with a positive association with DISH is *FGF2* which encodes a member of the fibroblast growth factor family and is involved in FGF signalling, which controls bone formation by regulating the expression of various genes involved in osteoblast differentiation and apoptosis [148], and thus abnormalities in this gene are closely related to ectopic ossification diseases. Disruption of the *FGF2* gene results in decreased bone mass and bone formation [384]. Jun and colleagues [147] found association between 2 polymorphisms in *FGF2* (rs1476217 and rs3747676) and DISH, however no other studies have confirmed this association. In our study we identified the 3'UTR variant rs1048201, which was heterozygous in three patients, however no differences were found between DISH/CC patients and controls. This variant (rs1048201) seems to contribute to osteoporosis susceptibility, most likely through their effects on altered binding affinity for specific miRNAs [385]. The 3'UTR region often contains regulatory elements that influence post transcriptional gene expression by MicroRNAs (miRNAs) [385, 386]. According to the miRNA related SNP database (miRNASNP v2.0, http://www.bioguo.org/miRNASNP2/online.php), this variant causes the loss of a binding site for hsa-miR-196a-3p miRNA. Probably in absence of the rs1048201 variant the has-miR-196a-3bp would bind optimally to FGF2 mRNA transcripts and negatively regulate protein expression by repressing mRNA translation and/or promoting mRNA degradation. On the other hand, the presence of rs1048201 variant may contribute to reduce binding efficacy between has-miR-196a-3bp and FGF2 and therefore would allow higher levels of FGF2 expression, which would be expected to stimulate osteoblastogenesis through osteoblasts formation and therefore bone formation. A recent study showed that the variant rs1048201 has also been reported to be associated with the risk of cleft palate [387], a disease which the etiopathogenesis is mostly unknown.

Normally, variants in the human genome with a deleterious effect on proteins are the basis for the development of diseases. However, all the *COL6A1* and *FGF2* variants reported in the literature with a significant association with DISH have a non-functional effect on protein and are common variants within the general population, which suggests that the gene variants found so far have a minor effect on the development of the disease. The findings of our study lead us to suggest, as described in OPLL [181], that DISH can also be influenced by the interaction of multiple gene variants in which *BMP4* gene could be one of them.

6.6. Conclusion

The variant rs17563 in *BMP4* gene was significantly associated with DISH/CC phenotype and may contribute to the development of DISH/CC phenotype. Further studies with a larger number of samples will be needed to clarify this association with the phenotype under study.

6.7. Future work

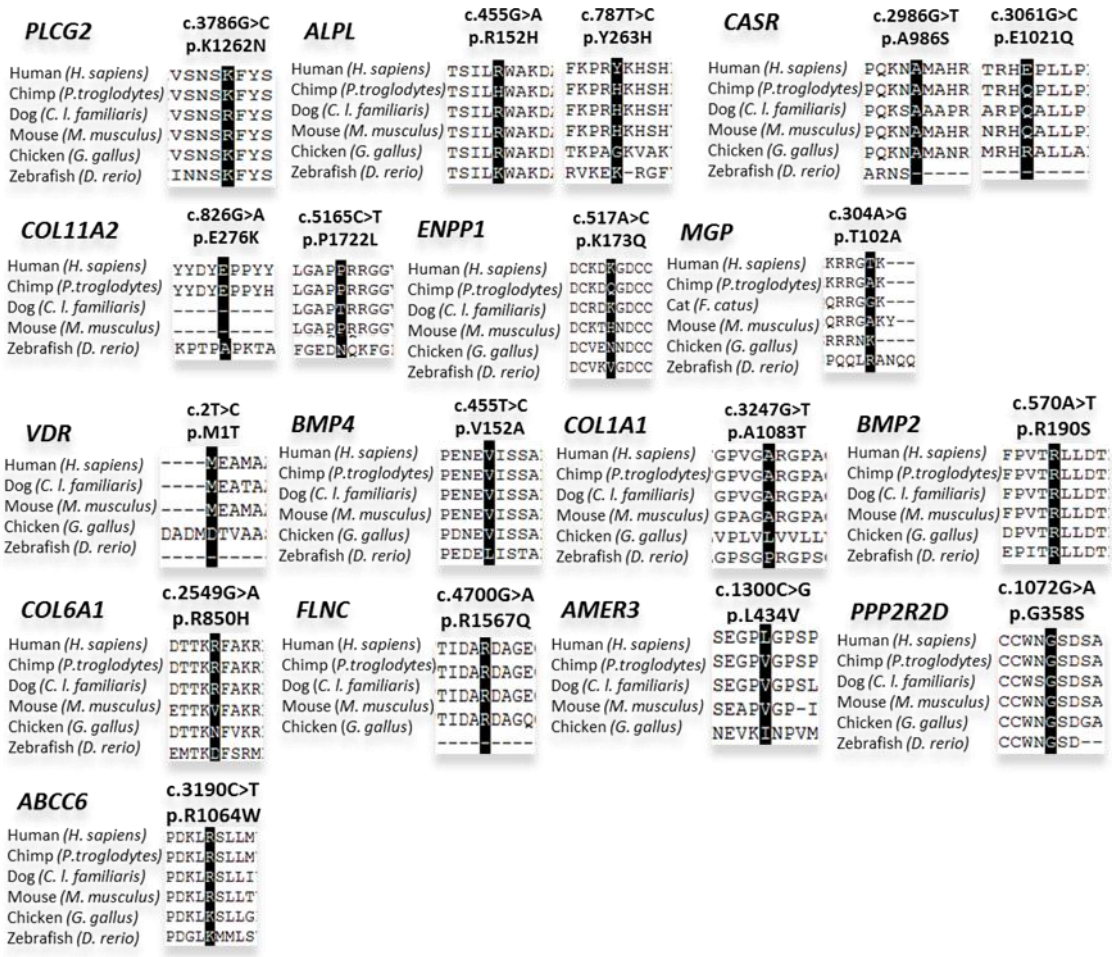
1) Explore how rs17563 in *BMP4* gene affects *BMP4* signaling in appropriate model systems.
2) Perform case/control studies in all the conserved genetic variants;
3) Investigate the role of HLA-DQA2 in this disorder by sequencing (typing) this gene in families and cohorts of patients/controls if necessary;
4) Design a customized panel (NGS) and by means of NGS sequence a cluster of genes involved in mineralization (including the genes identified in this study) in a cohort of patients and controls.

6.8. Supplementary material

Supplementary table 6-1. List of genes and their accession transcript numbers, used for conservation analysis. Data was retrieved from the Ensembl genome database; accessed on November 2016).

Genes	Specie		
	Human	**Chimp**	**Dog**
PLCG2	ENST00000564138.5	ENSPTRT00000015479.3	ENSCAFT00000031815.3
ALPL	ENST00000374840.7	ENSPTRT00000000592.2	ENSCAFT00000023578.3
CASR	ENST00000498619.3	ENSPTRT00000043996.4	ENSCAFT00000018760.3
COL11A2	ENST00000341947.6	ENSPTRT00000048564.4	ENSCAFT00000001409.4
ENPP1	ENST00000360971.6	ENSPTRT00000034356.3	ENSCAFT00000000001.3
MGP	ENST00000539261.5	ENSPTRT00000008732.4	ENSFCAT00000031714.1
VDR	ENST00000229022.7	Ni	ENSCAFT00000043473.2
BMP4	ENST00000245451.8	ENSPTRT00000011643.4	ENSCAFT00000023624.3
COL1A1	ENST00000225964.9	ENSPTRT00000017231.4	ENSCAFT00000026953.3
BMP2	ENST00000378827.4	ENSPTRT00000024606.3	ENSCAFT00000049690.1
COL6A1	ENST00000361866.7	ENSPTRT00000026156.3	ENSCAFT00000018918.3
FLNC	ENST00000325888.12	ENSPTRT00000036444.5	ENSCAFT00000002504.3
AMER3	ENST00000423981.1	ENSPTRT00000023124.3	ENSCAFT00000006747.2
PPP2R2D	ENST00000455566.5	ENSPTRT00000005829.4	ENSCAFT00000044416.1
ABCC6	ENST00000205557.11	ENSPTRT00000014398.4	ENSCAFT00000028908.3
Genes	**Specie**		
	Mouse	**Chicken**	**Zebrafish**
PLCG2	ENSMUST00000081232.8	ENSGALT00000050238.1	ENSDART00000021399.7
ALPL	ENSMUST00000030551.10	ENSGALT00000068960.1	ENSDART00000131101.2
CASR	ENSMUST00000063597.13	XM_416491.5.1	ENSDART00000010934.8
COL11A2	ENSMUST00000087497.10	Ni	ENSDART00000105754.4
ENPP1	ENSMUST00000105520.7	ENSGALT00000066498.1	ENSDART00000127350.3
MGP	ENSMUST00000032342.2	ENSGALT00000019173.4	ENSDART00000149622.2
VDR	ENSMUST00000023119.14	ENSGALT00000071682.1	ENSDART00000161892.1
BMP4	ENSMUST00000074077.11	ENSGALT00000020316.5	ENSDART00000075150.4
COL1A1	ENSMUST00000001547.7	XM_015273228.1.1	ENSDART00000009393.7
BMP2	ENSMUST00000028836.6	ENSGALT00000065435.1	ENSDART00000166657.1
COL6A1	ENSMUST00000001147.4	ENSGALT00000039669.3	ENSDART00000110608.3
FLNC	ENSMUST00000065090.7	NM_204573.1.1	Ni
AMER3	ENSMUST00000052670.10	ENSGALT00000055960.1	ENSDART00000149992.2
PPP2R2D	ENSMUST00000041097.12	ENSGALT00000081063.1	ENSDART00000172175.1
ABCC6	ENSMUST00000002850.7	ENSGALT00000048627.1	ENSDART00000172943.1

Abbreviations: Ni: note identified, *PLCG2*- Phospholipase C Gamma 2, *ALPL*- Alkaline Phosphatase, Liver/Bone/Kidney, *CASR*- calcium-sensing receptor, *COL11A2*- Collagen Type XI Alpha 2 Chain, *ENPP1*– ectonucleotide pyrophosphatase/phosphodiesterase 1, MGP- Matrix Gla Protein, VDR- Vitamin D Receptor, *BMP4*- Bone morphogenetic protein 4, *COL1A1*- Collagen Type I Alpha 1 Chain, *BMP2*- Bone morphogenetic protein 2, *COL6A1*- Collagen Type VI Alpha 1 Chain, *FLNC*- Filamin C, *AMER3*- APC Membrane Recruitment Protein 3, *PPP2R2D*- Protein Phosphatase 2 Regulatory Subunit B delta, *ABCC6* – ATP-binding cassete subfamily C, member 6.



Supplementary figure 6-1. Analysis of sequence conservation among six vertebrates (some genes are not available in all species) of variants identified in candidate genes possibly associated with DISH/CC phenotype. The variant for *FGF2* gene is not presented since it is a 3'prime region variant.

CHAPTER VII:
INVESTIGATING THE ROLE OF *ABCC6* GENE IN ECTOPIC CALCIFICATION

7. INVESTIGATING THE ROLE OF *ABCC6* GENE IN ECTOPIC CALCIFICATION

7.1 Abstract

Ectopic calcification is a pathological process caused by deposition of calcium salts or inappropriate biomineralization in soft tissues such as the eyes, skin, arteries, entheses, and cartilage. Both DISH/CC and Ankylosing Spondylitis (AS) are bone forming conditions that share many common features and often coexist, and therefore it has been proposed that their susceptibility genes may overlap. The *ABCC6* gene has been extensively related to dermal ectopic calcification and has recently become a new member of the calcification regulators in mammals. *ABCC6* deficiency causes an imbalance in the homeostasis of multiple organs leading to BMP activation and consequently bone formation in these sites. The present study investigates the role of the *ABCC6* gene in the aetiology of the following axial and peripheral ectopic calcification disorders: DISH, CC and AS. We test the hypothesis that if *ABCC6* is involved in DISH, CC and AS it's mutated in affected individuals. A preliminary expression study of the *ABCC6* gene was performed to verify gene expression and abundance in cartilage tissues.

DNA from 55 patients with DISH/CC, 25 with AS and 36 controls without DISH/CC and or AS were obtained for a case control association study. All 31 *ABCC6* exons and the proximal *ABCC6* gene promoter were amplified by PCR and Sanger sequencing of the products and statistical analysis was performed using Plink V1.9. *ABCC6* gene expression was performed using a quantitative RT-PCR (qPCR) in 13 cartilage samples obtained from the femoral head during hip replacements. The data analysis was performed using the comparative Ct method (ΔCt).

We identified 31 sequence variants of the *ABCC6* gene that were classified according to character into: regulatory (n = 3), missense (n = 6), splice site (n = 3), synonymous (n = 5), intronic (n = 13) and 1 variant located in the 3'UTR region. Using Cochran-Armitage trend adjusted for gender, the variant rs12931472 located in exon 14 of *ABCC6* gene was statistically more frequent in AS than in controls, but only in females. Furthermore, a gender-specific protective association of the rare variant rs41278174 located on exon 23 of the *ABCC6* gene was detected in both DISH/CC and AS males. When comparing DISH/CC with AS we observed that the regulatory variant rs778876717 was statistically more frequent in AS than in DISH/CC. The expression studies by qPCR showed that *ABCC6* transcripts was very

low abundance in in cartilage tissues, and in the patients with DISH and CC the gene was up-regulated in relation to a control patient.

Our findings support the hypothesis that the *ABCC6* gene plays a role in the axial ectopic calcification process and possibly reveals a gender specific interaction, however the statistical significance of our results is limited due to the relatively small cohort size, and larger cohort studies are required to further test this association. The gene expression studies on cartilage, although preliminary and not yielding statistically significant support reveals that differential expression could play a role in the disease process. To our knowledge, this is the first study to investigate the relationship between the *ABCC6* gene and diseases characterized by ossification of the axial skeleton, such as DISH and AS.

Keywords: *ABCC6*, DISH, CC, sequencing, qPCR.

7.2. Introduction

Ectopic calcification is caused by the inappropriate mineralization of soft tissues and is responsible for a significant number of disorders characterized by extraskeletal deposition of calcium and phosphate crystals [388]. Diffuse Idiopathic Skeletal Hyperostosis (DISH, MIM 106400), Chondrocalcinosis (CC, MIM 118600) and Ankylosing Spondylitis (AS, MIM 106300) are common rheumatic disorders characterized by ectopic calcification. DISH is characterized by the ossification of enthesis in the axial and peripheral skeleton, affecting the anterior longitudinal ligament [1] and CC by the deposition of calcium containing crystals in articular cartilage, synovial membranes and, less often, in periarticular soft tissues [341, 342]. AS is a chronic, multisystem inflammatory disorder characterized by inflammation and ankylosis of the sacroiliac joints and the axial skeleton. It is known that AS is strongly associated with HLA-B27 [235, 236], but other genes, including *ERAP1* and *IL23R*, have also been associated with the disease [243]. Studies of HLA association with DISH were performed due to the similarity of the radiographic images with AS. The association between DISH and the HLA locus is unclear and some studies have refuted the results [136-141], while others have demonstrated association [142, 143]. The discrepancy between the results obtained by different studies may be explained by the coincidence of DISH and AS, or by difficulties in differentiating between these two disorders. The association of HLA antigens with DISH has never been proven and this association has now been shelved. The aetiology of DISH is still unknown, but several lines of evidence suggest that genetic factors might be involved in its aetiology [64, 77, 78]. A small number of cases of monogenic CC (CC MIM

118600) are caused by mutations in the *ANKH* gene [110, 343-345], which encodes a multipass transmembrane protein ANK that transports intracellular inorganic pyrophosphate (PPi) to the extracellular milieu [106]. The coexistence of DISH with CC is very common on Terceira Island, Azores and it was hypothesized that both diseases share common pathogenic mechanisms [23].

Little is known about the reasons why calcification or ossification occurs outside the skeleton and considerable efforts have been made to understand why such events occur. Three proteins have been identified as central regulators of extracellular PPi and Pi levels, tissue-nonspecific alkaline phosphatase (TNAP) which converts the inhibitor (PPi) into a promoter of mineralization (Pi) [183], ectonucleotide pyrophosphatase 1 protein (ENPP1), which generates PPi from nucleoside triphosphates (ATP) [93] and the Inorganic Pyrophosphate Transport Regulator (ANK) which mediates intracellular to extracellular channelling of PPi [106]. Several other genes have been associated with the regulation of the biomineralization process, such as the *ABCC6* gene (ATP Binding Cassete Subfamily C Member 6), which maps to chromosome region 16p13.11 and encodes the transmembrane transporter MRP6 (Multidrug Resistance Protein 6) [389]. Inactivating mutations in this gene are the cause of most cases of Pseudoxanthoma elasticum (PXE; MIM#264800) and some cases of arterial calcification generalized of infancy type 2 (GACI2; MIM#614473)[390]. GACI is characterized by the calcification of the internal elastic lamina of muscular arteries and stenosis due to myointimal proliferation. Mutations in the *ENPP1* gene are the main cause for the GACI cases [96], and can also cause PXE [390]. In GACI the ectopic tissue mineralization occurs as a result of reduced ENPP1 activity and consequently an increase in the Pi to PPi ratio. PPi is hydrolyzed by TNAP to generate P_i, which is a component of hydroxyapatite crystal deposition and plays a role in the regulation of osteoblast differentiation [391]. PXE, in turn is characterized by aberrant mineralization of soft connective tissue with degeneration of the elastic fibers, involving primarily the eyes, the cardiovascular system, and the skin [392]. There have also been some studies associating polymorphisms in the *ENPP1* gene to a higher risk of developing ossification of the posterior longitudinal ligament (OPLL) [180].

There is a consensus that the *ABCC6* gene is mainly expressed in the liver and kidneys [393, 394], although two studies have reported its ubiquitous tissue expression [124, 395]. The majority of PXE causing mutations in *ABCC6* gene normally lead to an altered folding and/or protein stability leading to intracellular retention and reduced trafficking [112]. However, PXE is not caused by a lack of the functional MRP6 protein in the affected tissues but rather

by the absence of an unknown factor provided to the central circulation by an MRP6 dependent mechanism [396]. It is known that PPi is the central regulatory metabolite preventing matrix calcification in PXE, however the underlying molecular mechanism leading to reduction of PPi in PXE is unknown since the substrate(s) of this transporter remains to be elucidated. One theory suggested [129, 397] that MRP6 mediates the release of ATP directly from the liver into the circulation; ATP is converted into AMP and PPi and represents the main source of the mineralization inhibitor PPi in plasma. This explain why patients with PXE [397] and *Abcc6-/-* mice [129] have reduced PPi plasma levels compared to healthy individuals and wild type control mice, respectively. In the *Enpp1*$^{-/-}$ mouse model for GACI ectopic calcification also depends on plasma PPi levels and not on local PPi production [398]. The zebrafish mutant, *gräte* (*grt; abcc6ahu4958*) that carries a *abcc6a* mutated gene, shows signs of excessive hypermineralisation in the craniofacial and axial skeleton. In the zebrafish model the *abcc6a* gene is strongly expressed at the site of mineralisation and secretes ATP from cells increasing PPi locally, and this is unlike the mammalian model in which PPi is hepatically derived [113].

Although the PXE and GACI are considered two distinct diseases linked to *ABCC6* and *ENPP1* genes, respectively, the overlap of genotype and phenotype of both diseases suggests that the pathophysiology of both may derive from the same physiological mechanism or pathway [97, 390], probably involving reduced PPi [399]. Under physiological conditions PPi serves as a powerful anti-mineralization factor, preventing ectopic mineralization and dysregulated cellular production, degradation, and transport [400]. For instance, mutations in genes encoding known PPi-regulating enzymes like *ENPP1*, progressive ankylosis protein homolog (*ANKH*) and tissue-nonspecific alkaline phosphatase (*TNAP*) causes OPLL [195], CC [110] and Hypophosphatasia [401] , respectively. Furthermore, a recent study showed that polymorphisms in *ENPP1*, *TNAP* and *ANKH* are important genetic risk factors contributing to PXE [130].

The *ABCC6* gene has recently joined the list of calcification regulators as a new member playing a role in pyrophosphate metabolism. The present investigation is a follow-up study of the chapter 6 of this thesis, which includes the genetic analysis of all *ABCC6* gene (exons, the adjacent introns and the proximal promoter region). We test the hypothesis that if *ABCC6* is involved in DISH, CC and AS its expression will be modified in affected individuals. A preliminary expression study of the *ABCC6* gene was performed to verify gene expression and abundance in cartilage.

7.3. Material & methods

7.3.1. Subjects

This study involved a group of 12 probands (patients who are the initial members of a family to come under study) with a diagnosis of DISH/CC (8 male, 4 female; age of onset around 40 years), a cohort of 55 unrelated Azorean patients with a diagnosis of DISH/CC (36 male, 19 female; age of onset around 40 years), a cohort of 25 unrelated Azorean patients with a diagnosis of Ankylosing Spondylitis (18 male, 7 female; mean current age, 61 years; range, 37-91) and 36 unrelated healthy controls with a similar ethnic background (16 male, 20 female; mean current age, 68 years; range, 57-102). Standard X rays, to evaluate the severity status of the disease, were taken from: knees, axial skeleton, wrists, hands, elbows, and pelvis from selected DISH/CC patients.

In order to evaluate variant frequency two representative populations of Terceira Island, one with 124 individuals (45 male, 79 female; mean age, 66 years; range, 35-100) (population 1) and the other with 375 individuals (85 male, 29 female; mean age, 55 years; range, 25-90) (population 2) were used. This study was approved by the HSEIT Ethics Committee and all participants provided informed consent.

From 2008 to 2013 a collection of samples from the coxofemoral articular cartilage was obtained from 53 patients undergoing total hip replacement surgery (34 male, 17 female; mean current age, 71 years; range, 47-93 years). Sterile cartilage sections (of approximately 3 mm diameter) were prepared with a scalpel and a cutter and flash-frozen in RNA later at -80°C. Informed consent was obtained from these patients for use of their rejected tissue in research.

7.3.2. Gene sequencing

7.3.2.1. Mutation screening

Mutation screening was performed by Sanger sequencing of the 31 exons of *ABCC6*, including the exon–intron boundaries after polymerase chain reaction (PCR) amplification using previously described primers [308, 309], excluding the amplification of the two *ABCC6*-pseudogenes *ABCC6P1* and *ABCC6P2*, previously described [402] (primers sequences and PCR conditions available in the material and methods of this thesis). DNA from 12 DISH/CC probands was first sequenced followed by a group of 55 DISH/CC patients, 25 AS patients and 36 controls without DISH/CC disease.

Sequencing was carried out using ABI Big Dye chemistry on an ABI 3130xl automated sequencer (Applied Biosystems®) and genetic variants were screened with sequencing analysis and SeqScape (Applied Biosystems®) using as reference the NC_000016.10 sequence (see the material and methods section of this thesis for more detail).

The functional significance and the potential effect of each variant on the protein was subsequently explored in the following databases: dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP/, Human Gene Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk/ac/index.php), Human Splicing Finder (http://www.umd.be/HSF3/HSF.html), Ensembl (http://www.ensembl.org/index), making use of various algorithms, such as PolyPhen-2 (Polymorphism Phenotyping v2) (http://genetics.bwh.harvard.edu/pph2/) (benign, [0-0.2], possibly damaging (0.2-0.85), and probably damaging [0.85-1]) and SIFT (Sorting Intolerant From Tolerant) (damaging if less than 0.05). The Minor Allele Frequencies (MAF) of each variant were also analyzed.

Protein conservation analysis was performed using ClustalW (http://www.genome.jp/tools/clustalw/) to compare homologous amino acid sequences among multiple vertebrates at the sites where the variations occur. The accession numbers of each transcript are available in table 7-1.

Table 7-1. List accession transcript numbers for *ABCC6* gene, used for conservation analysis. Data was retrieved from the Ensembl genome database; accessed on Jully 2015.

Species	Accession numbers
Human	ENST00000205557.11
Chimpanzee	ENSPTRT00000014398.4
Mouse	ENSMUST00000002850.7
Cow	ENSBTAT00000050284.3
Dog	ENSCAFT00000028908.3
Chicken	ENSGALT00000048627.1
Xenopus	ENSXETT00000042610.2
Spotted gar	ENSLOCT00000008702.1

7.3.2.2. Statistical analysis

To assess the difference in allele frequencies between the 55 patients with DISH/CC and the 36 control individuals and between males and females a Fisher exact test was used. The same test was also used to assess the difference in the AS patients and controls. The Fisher exact test was calculated for odds ratios (OR) (95% confidence interval) and corresponding p-values.

To determine the association between the DISH/CC disease and allele variants other tests were employed: Cochran-Armitage trend, dominant and recessive gene action tests with 1 degree of freedom and genotypic test with 2 degrees of freedom. For all the statistical tests used a p-value of ≤0.05 was considered statistically significant. All statistical analysis were performed using PLINK software [354].

To compare the two groups of patients (DISH/CC and AS) a contingency table analysis for calculation of Fisher's exact probability test was performed using the VassarStats: Website for Statistical Computation (http://vassarstats.net/tab2x2.html).

7.3.3. Gene expression

7.3.3.1.RNA isolation and quality control

Cartilage samples were prepared using a combination of physical, chemical and enzymatic treatments. In brief, coxofemoral articular cartilage sections were minced, frozen, and pulverized in liquid nitrogen with a mortar and pestle. The tissue powder was placed in Trizol and total RNA was isolated using the Trizol RNA isolation protocol, developed by Chomezynski and Sacchi [310].

Purification of RNA samples was performed an RNeasy MinElute Cleanup kit (Qiagen) according to the manufacturer's instructions, a DNase (Deoxyribonuclease I) digestion and ethanol wash were subsequently performed. RNA integrity was assessed using a NanoVue spectrophotometer (GE Healthcare) and an Agilent 2100 Bioanalyser and RNA 6000 Nano LabChip (Agilent Technologies).

7.3.3.2 Reverse transcription- RT-PCR

RNA (100 ng) samples with RNA Integrity Number (RIN) above 4 were reverse-transcribed using High Capacity cDNA Reverse Transcription kit (Thermofisher) following the manufacturer's instructions. To verify the efficiency of RT-PCR the cDNA from human cartilage was used as a template for PCR amplification of the β-actin gene using a PCR System 9700 (Applied Biosystems). The β-actin amplicon of 249 bp was visualized on 1.7% agarose gel (sequence primers and PCR conditions available in material and methods of this thesis).

7.3.3.3.Quantitative RT-PCR (qRT-PCR)

Gene expression evaluation was performed using quantitative RT-PCR (qRT-PCR) with TaqMan chemistry. FAM-labeled TaqMan Gene Expression Assays (Applied Biosystems)

were used for the target gene *ABCC6* (*ABCC6*, Hs00184566_m1) and the primers include the 17-18 exon junction of this gene. The housekeeping gene *YWHAZ* (Tyrosine 3-Monnoxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta) is one of the most stable genes in cartilage tissues and is considered the best normalizer [403, 404] for use in this type of tissues. FAM-labeled TaqMan Gene Expression Assays (Applied Biosystems) were used for the internal control (*YWHAZ*, Hs00237047_m1), and the primers include the 1-2 exon junction of this gene.

The qPCR was performed for 20 µl final reactions using 20X TaqMan Gene Expression Master mix and 20X TaqMan Gene Expression assay for *ABCC6* and *YWHAZ* genes. Reactions were performed using the Applied Biosystems 7500 Fast Real-Time PCR System following the program: 2 min at 50°C, 10 min at 95°C, and 40 cycles repeating denaturation 15 s at 95°C, and annealing for 1 min at 60°C. Duplicate reactions were used for each sample and 400ng of synthesized cDNA was used to amplify the target gene and 200 ng to amplify the control gene.

Threshold cycle (Ct) values were determined using 7500 System Software V2.0.6 (Applied Biosystems) and data were further analyzed with Microsoft Office Excel 2013 (Microsoft Corporation). The data analysis was performed using the comparative Ct method (ΔCt) [405]. ΔCt values were used to measure gene expression, which was normalized using *YWHAZ* expression levels. The expression study was preliminary and because of low sample number it was not be possible to carry out statistical analysis.

7.3.3.4. Typing *ABCC6* gene variants

Functionally relevant variants were considered to be those that were located in regulatory regions such as; splice sites and missense variants in the promoter region, exons 10, 14,15, 22, 23, 25 and 27. The presence and location of mutations in the *ABCC6* gene were procured in the DNA of all patients selected for the expression studies (13 individuals; 9 males, 4 females; mean age of surgery, 66 years; range, 47-76 years).

7.4. Results

7.4.1. Sequencing

7.4.1.1. *ABCC6* variants

The coding region of the *ABCC6* gene was sequenced in a group of 12 DISH/CC probands and the variants identified are indicated in table 7-2. Thirty-one genetic variants were identified in the *ABCC6* gene; 3 located in regulatory regions, 3 in splice sites, 6 missense variants, 5 synonymous, 13 intronic and 1 variant located in the 3'UTR (Table 7-2).

Overall, three variants were found in the *ABCC6* promoter region variant rs28529549, and rs565625561 were present in P8 and rs778876717 were present in P1 and P9. The MAF values for the variants were not available in Ensembl. In the Human Gene Mutation Database the rs28529549 variant is associated with PXE and has a protective effect [406]. The splice site variants rs55778939 in P1, rs9940089 in P1-9 and P11 and rs41278172 in P8 probably have no impact on splicing. Splice variant rs41278172 is rare (MAF 0.01), rs55778939 is relatively rare (MAF 0.02) and rs9940089 is a frequent variant (MAF 0.18) (Table 7-2). Of the 6 missense variants, three of them are rare, with a MAF of 0.01. Variants rs41278174 in P1 and P9 and rs613450537 and rs2606921 in P8 were extremely rare and had an unknown MAF as there was insufficient data to establish population frequency since it was identified in only 3 of 114594 alleles (ExAc_Aggregated_Populations), indicating that it was unquestionably very rare (MAF 0.00003023). The variant rs41278174 (R1064W, P1 and P8) causes the substitution of arginine by tryptophan at amino acid 1064 in the protein and causes a shift from large and basic arginine to large and aromatic tryptophan.

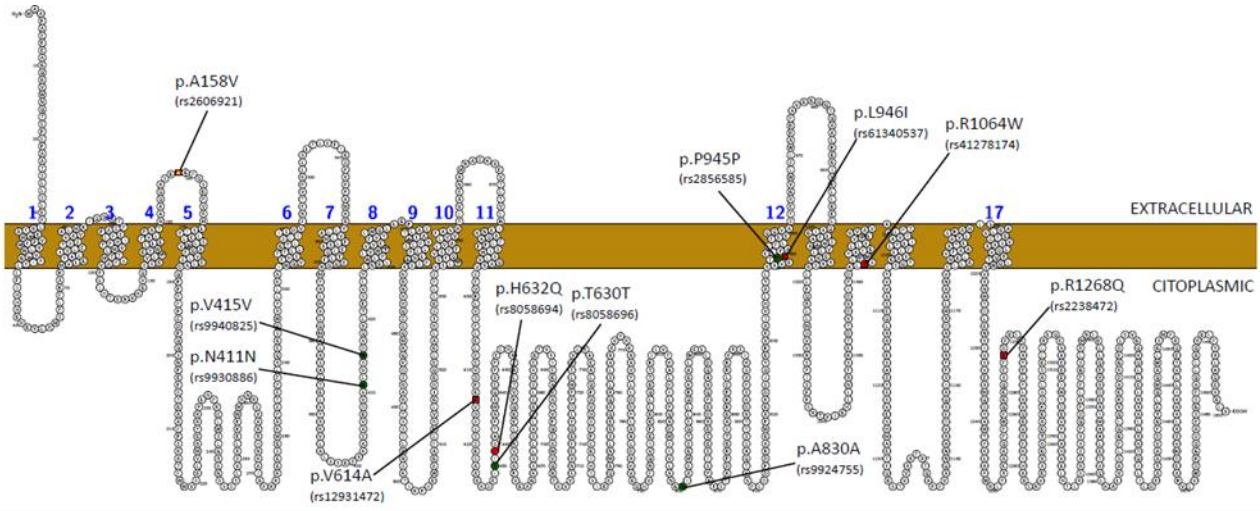
Table 7-2. *ABCC6* variants identified in a group of 12 DISH/CC probands (P1-12) and information about their functional significance (assessed on December 2016). The effect on splicing is indicated in Column SPL and absence of information stands for 'no affect splicing'.

Exon/ Intron modification	Variant	SNP	Type of variant	SPL	MAF	SIFT	PolyPhen	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
Promoter	c.-219A>C	rs28529549	RR	?	?	NA	NA								ht				
Promoter	c.-132C>T	rs565625561	RR	?	?	NA	NA								ht				
Promoter	c.-127C>T	rs778876717	RR	?	?	NA	NA	ht								hm			
intron3	c.345+26C>T	rs56019914	Intronic		0.03	NA	NA								ht				
i3	c.346-6G>A	rs55778939	Splicing		0.02	NA	NA	ht											
E4	c.473C>T (A158V)	rs2606921	Missense	as	?	0.22	0.003								ht				
i4	c.474+13G>A	rs111339199	Intronic		0.01	NA	NA	ht								ht			
i8	c.998+99G>A	rs150203830	Intronic		0.16	NA	NA	ht		ht	ht			?	ht		hm	ht	ht
i9	c.1176+243C>T	rs2283503	Intronic		0.36	NA	NA	ht	hm	ht	?					hm		ht	ht
i9	c.1177-94T>C	rs12935658	Intronic		0.32	NA	NA				ht	hm	hm	hm	ht				
i9	c.1177-89G>A	rs12935089	Intronic		0.32	NA	NA				ht	hm	hm	hm	ht				
E10	c.1233T>C (N411N)	rs9930886	Synonymous	as	0.28	NA	NA				ht	ht	ht	hm	ht				
E10	c.1245G>A (V415V)	rs9940825	Synonymous	as	0.23	NA	NA				ht	ht	ht	hm	ht				
i10	c.1338+7C>G	rs9940089	Splicing		0.18	NA	NA	ht	hm	ht	ht	hm	hm	hm	ht	ht		ht	
i10	c.1338+20C>T	rs12929920	Intronic		0.27	NA	NA	ht	hm	ht	ht	hm	ht	hm	ht	ht		ht	
i10	c.1338+62C>G	rs58394656	Intronic		0.17	NA	NA	ht		ht	ht	ht			ht	ht	hm	ht	hm
i11	c.1432-41A>G	rs2239322	Intronic		0.32	NA	NA	ht	ht	hm						hm		ht	
i12	c.1635+48C>T	rs55707615	Intronic		0.32	NA	NA	ht	ht	hm						hm		ht	
E14	c.1841T>C (V614A)	rs12931472	Missense		0.37	0.77	0	ht	ht		ht	ht	ht	hm	ht			ht	
i14	c.1868-57G>A	rs41278182	Intronic		0.30	NA	NA	ht	ht	hm						hm		ht	
E15	c.1890C>G T630T	rs8058696	Synonymous	as	0.33	NA	NA	ht	ht		ht	ht	hm	hm	ht			ht	
E15	c.1896C>A (H632Q)	rs8058694	Missense	as	0.36	0.59	0.001	ht	ht		ht	ht	hm	hm	ht			ht	
E19	c.2490C>T (A830A)	rs9924755	Synonymous	as	0.15	NA	NA	ht				ht	hm					ht	
E22	c.2835C>T (P945P)	rs2856585	Synonymous	as	0.12	NA	NA				ht								ht
E22	c.2836C>A (L946I)	rs61340537	Missense	as	0.01	0.42	0.013								ht				
E23	c.3190C>T (R1064W)	rs41278174	Missense	as	0.01	0	0.932	ht								ht			
i24	c.3507-3C>T	rs41278172	Splicing		0.01	NA	NA								ht				
E27	c.3803G>A (R1268Q)	rs2238472	Missense	as	0.19	0.19	0.004	ht	hm		hm			ht	ht	ht			ht
i28	c.4042-30C>T	rs2066738	Intronic		0.18	NA	NA		hm	ht						ht			
i30	c.4404-31A>G	rs212097	Intronic	as	0.28	NA	NA	ht	ht		hm	ht		ht	hm	ht	hm		hm
3'UTR	c.*17G>A	rs3902401	3'UTR	?	0.07	NA	NA								ht				ht

Abbreviations: SNP - Single nucleotide polymorphism, SPL- splicing, MAF – Minor Allele frequency, SIFT – Sorting Intolerant From Tolerant, P-proband, E-exon, i-intron, RR-Regulatory region, as - altered splicing NA - not applicable, ht - heterozygous, h - homozygous and ? - unknown genotype, as- altered splicing

According to the ensemble database this nucleotide position is totally conserved in mammals (Figure 7-1). This variant seems to have a fundamental relevance since its localization indicates a potential alteration of splicing and the algorithms SIFT score (0; deleterious) and PolyPhen (0.932; probably damaging) indicates a deleterious and a damaging effect on the protein (Table 7-2).

The positions of variants in the topology model of MRP6 protein and the conservation of the related amino acids are shown in figure 7-1. One variant occurs in extracellular domain (p.A158V), three variants in the transmembrane domain (P945P, L946I and R1064W), while seven were located in the cytoplasmic region (N411N, V415V, V614A, T630T, H632Q, A830A and R1268Q).



Protein region	Ext	CTD						TMD			CTD
AA change in human	A158V	N411N	V415V	V614A	T630T	H632Q	A830A	P945P	L946I	R1064W	R1268Q
Human	A	N	V	V	T	H	A	P	L	R	R
Chimpanzee	-	N	V	A	T	H	A	P	L	R	R
Mouse	G	N	V	G	S	H	T	P	L	R	R
Cow	G	N	V	A	S	Q	A	P	L	R	R
Dog	G	N	V	A	R	H	A	P	L	R	R
Chicken	R	N	V	-	I	R	T	P	L	K	R
Xenopus	-	N	T	S	T	R	R	W	Y	M	Q
Spotted gar	E	N	A	N	V	E	E	A	L	K	R

Figure 7-1. Predicted transmembrane protein topology of MRP6 (Protter) with locations of human variants and comparison with the amino acid in the equivalent position in other vertebrate species. In the protein structure the amino acids positions affected by a missense variant are highlighted in red and the synonymous variants are indicated in green. Ext: extracellular, CTD: cytoplasmatic domain and TMD: Transmembrane domain.

7.4.1.2. Case/control study

In order to perform a case/control study we selected functionally relevant variants, namely those that were located in regulatory regions including splice sites and missense variants and assessed their distribution in two groups of patients with DISH/CC and AS and in a group of unaffected controls. The 12 SNPs identified were in HWE and the results are shown in table 7-3. The missense variant rs12931472 in exon 14 was found in all AS females; 3 were heterozygous and 4 were homozygous (Supplementary table 7-1), however a fisher exact test did not give statistically significant p-value between AS females and controls females (Table 7-3). However, a significant difference was found between AS and control females when a Cochran-Armitage trend test (p=0.03, CHISQ=4.62) and allelic test (p=0.04, CHISQ=4.09) was used (Table 7-4). The missense variant rs41278174 in exon 23 was not found in the AS group (Supplementary table 7-1). Using the Cochran-Armitage trend and allelic tests the rs41278174 variant was significantly less frequent in both DISH/CC and AS males relative to the control males, where it was more frequent (Table 7-4).

Table 7-3. The results of the association study of genetic variants found in the *ABCC6* gene in Azorean patients with DISH/CC and AS compared to the controls. The minor allele is represented in bold.

SNP	Alleles	MAF									FISHER EXACT TEST											
		DISH/CC			CONTROLS			AS			DISH/CC vs Controls						AS vs Controls					
		All N=55	F N=19	M N=36	All N=36	F N=22	M N=14	All N=25	F N=7	M N=18	All		F		M		All		F		M	
											OR	P	OR	P	OR	P	OR	P	OR	P	OR	P
rs28529549	A/**C**	0.06	0.05	0.07	0.03	0	0.07	0.02	0	0.03	2.38	0.49	NA	0.21	0.97	1	0.71	1	NA	1	0.37	0.58
rs565625561	C/**T**	0.03	0	0.04	0.01	0	0.04	0.02	0	0.03	1.99	1	NA	1	1.17	1	1.45	1	NA	1	0.77	1
rs778876717	C/**T**	0.06	0.11	0.04	0.11	0.09	0.14	0.14	0.07	0.17	0.54	0.28	1.18	1	0.26	0.09	1.30	0.78	0.77	1	1.20	1
rs55778939	G/**A**	0.08	0.05	0.10	0.06	0.05	0.07	0.04	0.07	0.03	1.52	0.57	1.17	1	1.40	1	0.71	1	1.62	1	0.37	0.58
rs2606921	C/**T**	0.02	0	0.03	0.01	0	0.04	0.06	0	0.08	1.32	1	NA	1	0.77	1	4.53	0.30	NA	1	2.46	0.63
rs9940089	**C**/G	0.29	0.29	0.29	0.32	0.32	0.32	0.22	0.29	0.19	0.87	0.74	0.87	0.81	0.87	0.81	0.60	0.31	0.86	1	0.51	0.26
rs12931472	T/**C**	0.51	0.47	0.50	0.46	0.52	0.43	0.54	0.21	0.44	1.23	0.55	0.82	0.82	1.33	0.66	1.39	0.46	0.25	0.06	1.07	1
rs8058694	C/**A**	0.48	0.50	0.47	0.40	0.43	0.36	0.48	0.64	0.42	1.38	0.36	1.32	0.66	1.61	0.37	1.37	0.46	2.37	0.22	1.29	0.80
rs61340537	C/**A**	0.01	0	0.01	0.01	0.02	0	0.04	0	0.06	0.65	1	0	1	NA	1	2.96	0.57	0	1	NA	0.50
rs41278174	C/**T**	0.04	0.08	0.01	0.06	0.02	0.11	0	0	0	0.64	0.71	3.69	0.33	0.12	0.07	0	0.14	0	1	0	0.08
rs41278172	C/**T**	0.01	0	0.01	0.01	0.02	0	0.04	0	0.06	0.65	1	0	1	NA	1	2.96	0.57	0	2.92	NA	0.50
rs2238472	G/**A**	0.22	0.24	0.21	0.21	0.20	0.21	0.24	0.43	0.17	1.06	1	1.21	0.79	0.96	1	1.20	0.82	2.92	0.16	0.73	0.75

Abbreviations: SNP- Single nucleotide polymorphism, AS- Ankylosing Spondylitis, MAF- Minor allele frequency, OR- odds ratio, P- pvalue, N- number of individuals, NA- not applicable, vs: versus, F- Female, M- Male.

Table 7-4. Association study of the genetic variants in the *ABCC6* gene in DISH/CC and AS patients compared to the controls.

SNP	Allele (A1)	Allele (A2)	DISH/CC (A1/A2)			Controls (A1/A2)			AS (A1/A2)			TEST	DISH/CC vs Controls						AS vs Controls					
													All		M		F		All		M		F	
			All N=110	M N=72	F N=38	All N=72	M N=28	F N=44	All N=50	M N=36	F N=14		CHISQ	P	CHISQ	P	CHISQ	P	CHISQ	P	CHISQ	P	CHISQ	P
rs28529549	C	A	7/103	5/67	2/36	2/70	2/26	0/44	1/49	1/35	0/14	T	1.26	0.26	0	0.97	2.44	0.12	0.08	0.78	0.71	0.40	NA	NA
												A	1.19	0.28	0	0.97	2.37	0.12	0.07	0.79	0.67	0.41	NA	NA
rs565625561	T	C	3/107	3/69	0/38	1/71	1/27	0/44	1/49	1/35	0/14	T	0.37	0.54	0.02	0.89	NA	NA	0.07	0.79	0.03	0.85	NA	NA
												A	0.36	0.55	0.02	0.89	NA	NA	0.07	0.79	0.03	0.86	NA	NA
rs778876717	T	C	7/103	3/69	4/34	8/64	4/24	4/40	7/43	6/30	1/13	T	1.43	0.23	3.43	0.06	0.05	0.82	0.27	0.61	0.08	0.77	0.06	0.81
												A	1.30	0.25	3.17	0.07	0.05	0.83	0.23	0.63	0.07	0.79	0.05	0.82
rs55778939	A	G	9/101	7/65	2/36	4/68	2/26	2/42	2/48	1/35	1/13	T	0.42	0.52	0.14	0.71	0.02	0.88	0.16	0.69	0.71	0.40	0.15	0.69
												A	0.45	0.50	0.16	0.69	0.02	0.88	0.15	0.70	0.67	0.41	0.15	0.70
rs2606921	T	C	2/108	2/70	0/38	1/71	1/27	0/44	3/47	3/33	0/14	T	0.05	0.82	0.05	0.83	NA	NA	2.05	0.15	0.65	0.42	NA	NA
												A	0.05	0.82	0.04	0.86	NA	NA	1.98	0.16	0.61	0.44	NA	NA
rs9940089	C	G	32/78	21/51	11/27	23/49	9/19	14/30	11/39	7/29	4/10	T	0.15	0.70	0.08	0.78	0.06	0.80	1.23	0.27	1.35	0.24	0.04	0.84
												A	0.17	0.68	0.09	0.77	0.08	0.78	1.45	0.23	1.35	0.24	0.05	0.82
rs12931472	T	C	56/54	36/36	18/20	33/39	12/16	23/21	27/23	16/20	3/11	T	0.46	0.50	0.40	0.53	0.22	0.64	0.89	0.35	0.02	0.89	**4.62**	**0.03**
												A	0.45	0.50	0.41	0.52	0.20	0.66	0.79	0.37	0.02	0.90	**4.09**	**0.04**
rs8058694	A	C	53/57	34/38	19/19	29/43	10/18	19/25	24/26	15/21	9/5	T	1.07	0.30	1.06	0.30	0.37	0.54	0.74	0.39	0.27	0.61	1.83	0.18
												A	1.10	0.29	1.08	0.30	0.38	0.54	0.72	0.40	0.23	0.63	1.89	0.17
rs61340537	A	C	1/109	1/71	0/38	1/71	0/28	1/43	2/48	2/34	0/14	T	0.09	0.76	0.40	0.53	0.89	0.35	0.86	0.35	1.66	0.20	0.33	0.57
												A	0.09	0.76	0.39	0.53	0.87	0.35	0.84	0.36	1.61	0.21	0.32	0.57
rs41278174	T	C	4/106	1/71	3/35	4/68	3/25	1/43	0/50	0/36	0/14	T	0.40	0.53	**4.76**	**0.03**	1.46	0.23	2.97	0.08	**4.26**	**0.04**	0.33	0.57
												A	0.38	0.54	**4.57**	**0.03**	1.39	0.24	2.87	0.09	**4.05**	**0.04**	0.32	0.57
rs41278172	T	C	1/109	1/71	0/38	1/71	0/28	1/43	2/48	2/34	0/14	T	0.09	0.76	0.40	0.53	0.89	0.35	0.86	0.35	4.66	0.20	0.33	0.57
												A	0.09	0.76	0.39	0.53	0.87	0.35	0.84	0.36	1.61	0.21	0.321	0.57
rs2238472	A	G	24/86	15/57	9/29	15/57	6/22	9/35	12/38	6/30	6/8	T	0.02	0.88	0	0.95	0.12	0.73	0.17	0.69	0.24	0.62	2.75	0.10
												A	0.03	0.87	0	0.95	0.12	0.72	0.17	0.68	0.23	0.63	2.78	0.10

Abbreviations: SNP- Single nucleotide polymorphism, AS- Ankylosing Spondylitis**,** M-Males, F-females, T-trend, A- allelic, P- p-value, N- number of individuals.

To compare the variant frequency between DISH/CC and AS we used a Fisher Exact Probability test (Table 7-5).

Table 7-5. Frequency comparison of the genetic variants found in the coding exons of the *ABCC6* gene between the DISH/CC and AS groups.

SNP	Allele (A1)	Allele (A2)	DISH/CC (A1/A2)			AS (A1/A2)			p-value one tailed/two tailed		
			All N=110	M N=72	F N=38	All N=50	M N=36	F N=14	All	M	F
rs28529549	C	A	7/103	5/67	2/36	1/49	1/35	0/14	0.22/0.44	0.34/0.66	0.53/1
rs565625561	T	C	3/107	3/69	0/38	1/49	1/35	0/14	0.62/1	0.59/1	1/1
rs778876717	T	C	7/103	3/69	4/34	7/43	6/30	1/13	0.10/0.13	**0.03/0.05**	0.59/1
rs55778939	A	G	9/101	7/65	2/36	2/48	1/35	1/13	0.27/0.50	0.18/0.26	0.61/1
rs2606921	T	C	2/108	2/70	0/38	3/47	3/33	0/14	0.18/0.33	1/1	0.21/0.33
rs9940089	C	G	32/78	21/51	11/27	11/39	7/29	4/10	0.23/0.44	0.20/0.35	0.63/1
rs12931472	T	C	56/54	36/36	18/20	27/23	16/20	3/11	0.42/0.74	0.37/0.68	0.08/0.11
rs8058694	A	C	53/57	34/38	19/19	24/26	15/21	9/5	0.56/1	0.37/0.68	0.27/0.53
rs61340537	A	C	1/109	1/71	0/38	2/48	2/34	0/14	0.23/0.23	0.67/1	1/1
rs41278174	T	C	4/106	1/71	3/35	0/50	0/36	0/14	0.22/0.31	0.66/1	0.21/0.31
rs41278172	T	C	1/109	1/71	0/38	2/48	2/34	0/14	0.23/0.23	0.26/0.55	1/1
rs2238472	A	G	24/86	15/57	9/29	12/38	6/30	6/8	0.45/0.84	0.41/0.80	0.16/0.30

Abbreviations: SNP- Single nucleotide polymorphism, AS- Ankylosing Spondylitis**,** M-Males, F-females, T-trend, A- allelic, OR- odds ratio, P- pvalue, N- number of individuals.

The frequency of the variant rs778876717 was significantly different between the DISH/CC and AS cohorts, particularly in males and it was more frequent in AS males than in DISH/CC males (Table 7-5). The rs778876717variant is located in the promotor region and no frequency or population information exists for this variant.

7.4.1.3 SNP frequencies

The variant rs41278174 (R1064W) was found in ten individuals (in two probands, 4 DISH/CC patients and 4 healthy controls). Since rs41278174 is extremely rare (Table 7-2) we procured for this variant in two representative populations of Terceira Island that contained 124 and 385 individuals to verify if the frequency is also higher in these groups (Table 7-6).

Table 7-6. Frequency in population 1 and population 2 of Terceira Island of the *ABCC6* gene variant (c.3190C>T/R1064W) in exon 23.

Variant	SNP	Population 1			Population 2		
		All (N=124)	F (N=77)	M (N=45)	All (N=385)	F (N=299)	M (N=86)
c.3190C>T R1064W	rs41278174	14 11%	11 14%	3 7%	19 5%	16 5%	3 3%

Abbreviations: SNP- Single nucleotide polymorphism, F-females, M-Males, N- number of individuals.

The c.3190C>T/R1064W variant was found in 14 individuals in population 1 and in 19 individuals in population 2, all of them were heterozygous. In addition, a further rare variant (rs60707953) was found in exon 23, which have a MAF of 0.01 in two individuals, one female and one male from the population 1. The male suffers from lower back pain and the female is healthy. The variant causes a substitution of a leucine by isoleucine at amino acid 1097 in the protein. Both amino acids have similar physico-chemical properties and both residues are medium size and hydrophobic.

7.4.2. Expression

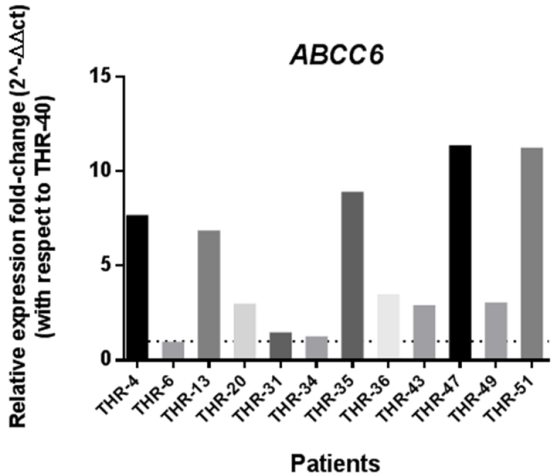
It was only possible to assess the expression of the *ABCC6* gene in 13 (9 males and 4 females; mean age 72 years; range 53-84) (Table 7-7 and supplementary figure 7.1) out of 53 RNA cartilage samples due to the poor quality of the RNA (Supplementary table 7-2).

Table 7-7. Details of the 13 patients undergoing hip replacement from whom femoral head cartilage where obtained. The sample used as a control is highlighted in bold (THR-40). * Age (yr-years) at time of operation. The pathological anatomy of the affected tissue is also indicated.

Samples	Sex	Age (yr)*	Diagnosis	Affected tissue
THR-4	F	61(?)	DISH (thoracic)	Cartilaginous tissue
THR-6	M	67	Coxoarthrosis (right)	Cartilaginous tissue with degenerative alterations
THR-13	M	76	DISH (cervical, lumbar and throracic), Coxoarthrosis	Cartilaginous tissue
THR-20	M	70	Coxoarthrosis (bilateral), hypertension	Cartilaginous tissue with superficial fibrosis with papilliform contours
THR-31	M	47	Coxoarthrosis (right)	Fragment of cartilaginous tissue with focal fibrous cap
THR-34	F	58	Coxoarthrosis (left)	Synovial and cartilaginous fibrous tissue
THR-35	M	75	Chondrocalcinosis (knees), Coxoarthrosis (bilateral)	Cartilaginous tissue
THR-36	F	59	Chondrocalcinosis (knees), Coxoarthrosis	Cartilaginous tissue with degenerative aspects
THR-40	**F**	**64**	**Left femoral fracture**	**No pathology**
THR-43	M	73	Osteoarthritis	Cartilaginous tissue
THR-47	M	64	Coxoarthrosis (left), lithiasis	Cartilaginous tissue with superficial fibrosis forming papillary projections
THR-49	M	76	Coxoarthrosis (right)	Fragments of cartilaginous tissue with calcifications
THR-51	M	63	Coxoarthrosis (right), Rheumatoid arthritis	Cartilaginous tissue

Abbreviations: F-females, M-Males, THR: Total hip replacement.

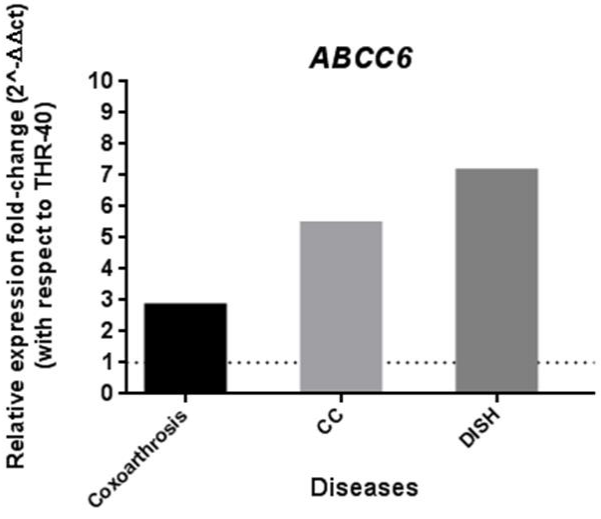
Of the 13 samples selected for expression studies (9 males, 4 females; mean age at surgery, 66 years; range, 47-76 years) there were two patients with DISH (confirmed with radiology), two patients with Knee chondrocalcinosis, 1 with lithiasis, 1 with osteoarthritis and 1 with rheumatoid arthritis (Table 7-7). These 5 groups of patients were compared with the control sample THR-40, since this individual did not present any disease pathology characterized by ectopic calcifications and the medical intervention was only due to a femoral fracture. Gene transcripts of the *ABCC6* gene were detected in cartilage tissues. The *ABCC6* gene was up regulated in all samples from diseased tissue relative to the control sample THR-40. The only exception was THR-6 patient in which *ABCC6* transcript abundance was lower than the control sample (Figure 7-2). The samples with higher *ABCC6* transcripts were THR-4, THR-13, THR-35, THR-47 and THR-51.



Patients	THR-4	THR-6	THR-13	THR-20	THR-31	THR-34	THR-35	THR-36	THR-43	THR-47	THR-49	THR-51
Relative expression	7,57	0,87	6,74	2,88	1,37	1,15	8,80	3,39	2,80	11,25	2,94	11,12

Figure 7-2. Relative expression of *ABCC6* gene in cartilage tissue samples. The expression of the *ABCC6* gene was determined in cDNA synthesized from cartilage tissues. The values were normalized in relation to the expression of the reference gene *YWHAZ*. The dashed line in the graph represents the value 1 of the control sample TRH40.

Analyzing the samples by grouping them according to pathology revealed that the groups with coxoarthrosis, chondrocalcinosis and DISH, seemed to have a higher expression of the *ABCC6* gene in relation to the normal control THR-40, but the DISH group is the group with more expression (Figure 7-3).



Diseases	Coxoarthrosis	CC	DISH
Relative expression	2,83	5,46	7,14

Figure 7-3. Relative expression of the *ABCC6* gene in normal (n = 1), coxoarthrosis (n = 8), DISH (n = 2) and CC (n = 2) individuals. Coxoarthrosis group include all the THR cohort with the exception of the normal individual THR-40 and the CC (THR-35, 36) and DISH patients (THR-4, 13).

7.5. Discussion

Both, DISH and AS are bone forming diseases that involve the axial skeleton and the peripheral entheses, resulting in bone proliferation in the spine and at the extraspinal entheseal sites in the later phases of their course. Since these conditions can coexist it has been suggested that their susceptibility genes may overlap. We performed a mutational analysis of the *ABCC6* gene in a group of DISH/CC and AS patients in order to find a possible association between the gene and these diseases. In this study we identified 31 different variants along the 31 exons of *ABCC6* gene. Analysis of the topology of these variants in the MRP6 protein (Figure 7-1) shows that the vast majority lie within the cytoplasmic domains. In PXE diseases the majority of disease causing variants are also located in the cytoplasmic region, particularly in the eighth intracellular loop encoded by exon 24, and the two nucleotide binding folds (NFB1 and NFB2) of MRP6, encoded by the exons 16-18 and 28-30, respectively [407]. As expected, in our group of patients that did not present PXE lesions, we did not find variants in these particular cytoplasmic regions. However in general it is thought that missense variants within the cytosolic domain of proteins, may affect their function by changing their substrate specificity or by disrupting the correct folding of the protein necessary for its function [308]. The variant rs12931472 of the *ABCC6* gene is located in the sixth intracellular loop of the deduced protein, and is encoded by exon 14 and causes the substitution of amino acid 614 from a medium size and hydrophobic valine (V) to a small size and hydrophobic alanine (A). As shown in figure 7-1 this amino acid position is variable in other species, furthermore the altered amino acid (A) in the human variant is the normal amino acid in other vertebrates, such as chimpanzee, cow and dog. This variant is frequent in the human *ABCC6* gene and it has a MAF value of 0.34(G) in all populations and in Peruvian population the presence of this variant can reach to 52%. In the present study the rs12931472 variant was not associated with the occurrence of the DISH/CC or AS phenotypes; however a Cochran-Armitage trend and allelic tests adjusted for gender indicated that this variant is statistically more frequent in AS females than in control females. Despite the limited number of AS females (n=14) in the present study all of them had the rs12931472 variation in a heterozygous or homozygous state. The sexual divergence in *ABCC6* variants ties in with the notion that gender specific differences occur between males and females with AS. AS females are more prone to cervical spine involvement, and males frequently complain about lumbar pain [408]. It is observed that female AS patients present higher fatigue scores, higher disease activity and more frequent peripheral involvement [409, 410]. Different haplotype

combinations in the *ANKH* gene have also been reported in males and females with AS. The authors found that in families with affected individuals of both sexes, two *ANKH* SNPs (rs28006 and rs25957) were associated with AS but only in affected women [248]. It is unlikely that the major genetic factors that account for these differences are X-linked because there is no linkage of AS susceptibility with X-chromosome markers [411]. The underlying pathogenic mechanism that result in gender-associated differences in terms of the manifestations of AS, is currently unknown.

The missense variant rs41278174 identified in this study is rare (MAF 0.01) and is located on transmembrane domain; comparative analysis revealed that the modified amino acid is normally highly conserved between mammals (Figure 7-1) and although SIFT and PolyPhen indicate the mutation is deleterious it has never been linked to PXE or other disorder. The variant was found in two PXE families, however it was considered non disease causing since it did not segregate with the disease phenotype [308]. We also evaluated the allele frequency of this rare variant in two groups of Azorean Terceira island individuals with 124 and 385 individuals. The variant was detected in 33 individuals, indicating that this variant is not as rare in the island as in the general population from 1000 genomes project. The higher frequency of this variant in Terceira island (11% in population 1) can be explained by the consanguinity which tends to occur on small islands. Besides, this variant was not found in the AS group and it was significantly more frequent in male controls than in DISH/CC and AS males, which may suggest it has a protective effect against both diseases, but only in males. The protective effect of this variant on both diseases is difficult to explain and may suggest it affects pathological features shared by DISH and AS, such as the ossification of the axial skeleton. It is known that the transmembrane domain of MRP6 determines the substrate specificity of ABC transporters, and therefore it is likely that missense variants in this region change the substrate specificity of MRP6 causing substrate accumulation [412]. Mutations in the transmembrane domain can also affect the integration of the protein into the cell membrane and lead to a loss of function [308]. Probably the variant rs41278174 in males changes the specificity of MRP6 transporter, conferring a protective effect via an unknown mechanism.

In the *ABCC6* promoter we found the polymorphisms rs28529549, rs565625561 and rs778876717. Interestingly the rs28529549 (c.-219A>C) variant, according to Schulz et al [406], has a protective effect for PXE disease and is located in a transcriptional activator sequence of the *ABCC6* promoter and occurs in the binding site of a transcriptional repressor, predominantly found in genes that participate in lipid metabolism. In fact, several studies

have described genetic mutations in the *ABCC6* gene associated with variations in quantitative plasma lipoproteins [413], low High density lipoprotein (HDL) and/or coronary heart disease risk [414]. Alterations in lipoprotein composition with lowered plasma HDL cholesterol levels and hypertriglyceridemia were also found in plasma samples of PXE patients [406]. Furthermore, the *Abcc6* knockout mice have HDL cholesterol plasma levels reduced by 25% [415], confirming the potential role of *ABCC6* in lipid homeostasis [416]. The association between the polymorphism rs2238472 (p.R1268Q) in the exon 27 of the *ABCC6* gene, found in our study in a great number of individuals (7 probands, 10 AS; 14 controls and 14 DISH/CC individuals), with plasma lipoprotein levels has previously been reported [413]. The variant is pathogenic (HGMD mutation) for PXE disease only in compound heterozygous (associated with R1141X) and is harmless in homozygotes [417].

Coxoarthrosis is characterized by focal areas of damage to the hip articular cartilage, associated with increased activity in the subchondral bone and marginal osteophyte formation [418], leading to hip replacement surgery in majority of cases. The most common reason for hip replacement is osteoarthritis, but other diseases such as rheumatoid arthritis [419], DISH [420] and CC [421, 422] can also be responsible for this outcome. In the present study in general the *ABCC6* gene had expression in cartilage tissues and its expression was much higher in DISH/CC patients. As already mentioned, it is believed that the MRP6 mediates ectopic calcification via the circulation since *ABCC6* is absent or minimally expressed in the calcified tissue resulting from the deficiency [423].

Previous studies on *ABCC6* associate BMP signaling with *ABCC6* deficiency and it is this that promotes the osteochondrogenic transitions in susceptible cells [423]. One study revealed BMP2/SMADS/RUNX2 were up-regulated as were the Tgfb2/smad2/3 pathways at the mineralization sites in *ABCC6* deficiency mice [424]. The *ABCC6* deficient mice develops ectopic calcification similar to both the human PXE and mouse dystrophic cardiac calcification phenotypes, and in mice this *ABCC6* deficiency stimulates BMP signaling by acting on the expression of multiple BMPs. The same authors verified an enhanced BMP signaling in liver, kidney and aortic tissues in the absence of *ABCC6*, leading the authors to hypothesize that deficiency of ABCC6, which is mainly expressed in liver, affects BMP signaling in other organs [423]. It is believed that the change in BMP signaling involves matrix Gla protein (MGP), an extracellular glycoprotein that inhibits tissue mineralization by chelating calcium [425] and regulating local BMP signaling [426]. However, to be a calcification inhibitor, MGP has to be activated by γ-glutamyl carboxylation, a vitamin k dependent reaction. This protein is active in its carboxylated form (cMGP) and inactive in the

un-carboxylated form (ucMGP) [121]. In one study MGP was isolated from the liver of Abcc6-/- mice and was largely present as ucMGP and was inactive, and it was demonstrated that there is an association between ucMGP forms of MGP and ectopic mineralization [427]. Low levels of serum MGP have been reported in MRP6 deficiency and PXE patients have also lower serum concentration of MGP compared to controls, MGP is present within elastic fibers and is associated with calcification of dermal elastic fibers [428, 429]. MGP is expressed in various tissues and up regulated in bone and cartilage [430], it is believed that MGPs actions include the inhibition of mineralization by suppression of *BMP2*, which is a potent osteogenic factor [431]. Sarzi-Puttini et al reported higher serum MGP levels in DISH males and females, and the group suggested that MGP may be a marker of hyperostosis because it is produced in larger amounts by this patients [432]. It is possible that the higher concentration of ucMGP reported in DISH patient is most likely a consequence of the failure of the method to distinguish between the noncarboxylated and the γ- carboxylated forms of MGP. Other investigations have shown that BMP2 can regulate phosphate uptake [433], and that phosphate levels can regulate *BMP2* expression [434], however it is not clear how pyrophosphate mediates other MRP6 actions. Although the physiological function of MRP6 towards calcification is likely exerted via the systemic circulation of its substrate(s), the exact mechanism by which this protein affects the susceptibility to mineralization in distal tissues has yet to be defined.

Our results suggest that *ABCC6* gene plays a role in the ectopic calcification process in patients from the Azores and identifies possible alleles and reveals probable gender specificity in this interaction. The expression study of *ABCC6* in cartilage suggests that differential expression of *ABCC6* transcripts could play a role in the disease process. However, a much bigger sample size will be required to give robust statistical support to this hypothesis.

7.6. Supplementary material

Supplementary table 7-1. Results of genetic variants found in the *ABCC6* gene in Azorean patients with DISH/CC and AS compared to the controls.

SNP	Genotype	DISH/CC			SPA			Controls		
		All: N=55			All: N=25			All: N=36		
		Female: N=19			Female: N=7			Female: N=22		
		Male: N=36			Male: N=18			Male: N=14		
		All N (%)	Female N (%)	Male N (%)	All N (%)	Female N (%)	Male N (%)	All N (%)	Female N (%)	Male N (%)
rs28529549	AA	48 (87)	17 (89)	31 (86)	24 (96)	7 (100)	17 (94)	34 (94)	22 (100)	12 (86)
	AC	7 (13)	2 (10)	5 (14)	1 (4)	0 (0)	1 (6)	2 (6)	0 (0)	2 (14)
	CC	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
rs565625561	CC	52 (95)	19 (100)	33 (92)	24 (96)	7 (100)	17 (94)	35 (97)	22 (100)	13 (93)
	CT	3 (5)	0 (0)	3 (8)	1 (4)	0 (0)	1 (6)	1 (3)	0 (0)	1 (7)
	TT	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
rs778876717	CC	48 (87)	15 (79)	33 (92)	18 (72)	6 (86)	12 (67)	28 (78)	18 (82)	10 (71)
	CT	7 (13)	4 (21)	3 (8)	7 (28)	1 (14)	6 (33)	8 (22)	4 (18)	4 (29)
	TT	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
rs55778939	GG	47 (85)	17 (89)	30 (83)	23 (92)	6 (85)	17 (94)	32 (89)	20 (91)	12 (86)
	GA	7 (13)	2 (11)	5 (14)	2 (8)	1 (14)	1 (6)	4 (11)	2 (9)	2 (14)
	AA	1 (2)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
rs2606921	CC	53 (96)	19 (100)	34 (94)	22 (88)	7 (100)	15 (83)	35 (97)	22 (100)	13 (93)
	CT	2 (4)	0 (0)	2 (6)	3 (12)	0 (0)	3 (17)	1 (3)	0 (0)	1 (7)
	TT	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
rs9940089	CC	5 (9)	10 (52)	18 (50)	1 (4)	4 (57)	11 (61)	6 (17)	12 (55)	7 (50)
	CA	22 (40)	7 (37)	15 (42)	9 (36)	2 (29)	7 (39)	11 (31)	6 (27)	5 (36)
	AA	28 (51)	2 (11)	3 (8)	15 (60)	1 (14)	0 (0)	19 (53)	4 (18)	2 (14)
rs12931472	TT	13 (24)	4 (21)	9 (25)	4 (16)	0 (0)	4 (22)	10 (28)	5 (23)	5 (36)
	TC	28 (51)	10 (53)	18 (50)	15 (60)	3 (43)	12 (67)	19 (53)	13 (59)	6 (43)
	CC	14 (25)	5 (26)	9 (25)	6 (24)	4 (57)	2 (11)	7 (19)	4 (18)	3 (21)
rs8058694	CC	15 (27)	5 (26)	10 (28)	6 (24)	1 (14)	5 (28)	13 (36)	7 (32)	6 (43)
	CA	27 (49)	9 (47)	18 (50)	14 (56)	3 (43)	11 (61)	17 (47)	11 (50)	6 (43)
	AA	13 (24)	5 (26)	8 (22)	5 (20)	3 (43)	2 (11)	6 (17)	4 (18)	2 (14)
rs61340537	CC	54 (98)	19 (100)	35 (97)	23 (92)	7 (100)	16 (89)	35 (97)	22 (100)	13 (93)
	CA	1 (2)	0 (0)	1 (3)	2 (8)	0 (0)	2 (11)	1 (3)	0 (0)	1 (7)
	AA	0 (0)	0 (0)	0 (0)	2 (8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
rs41278174	CC	51 (93)	16 (84)	35 (97)	25 (100)	7 (100)	18 (100)	32 (89)	21 (95)	11 (79)
	CT	4 (7)	3 (16)	1 (3)	0 (0)	0 (0)	0 (0)	4 (11)	1 (4)	3 (21)
	TT	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
rs41278172	CC	54 (98)	19 (100)	35 (97)	23 (92)	7 (100)	16 (89)	35 (97)	21 (95)	14 (100)
	CT	1 (2)	0 (0)	1 (3)	2 (8)	0 (0)	2 (11)	1 (3)	1 (4)	0 (0)
	TT	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
rs2238472	GG	35 (64)	12 (63)	23 (64)	15 (60)	3 (43)	12 (67)	22 (61)	13 (59)	9 (64)
	GA	16 (29)	5 (26)	11 (31)	8 (32)	2 (29)	6 (33)	13 (36)	9 (41)	4 (29)
	AA	4 (7)	2 (11)	2 (6)	2 (8)	2 (29)	0 (0)	1 (3)	0 (0)	1 (7)

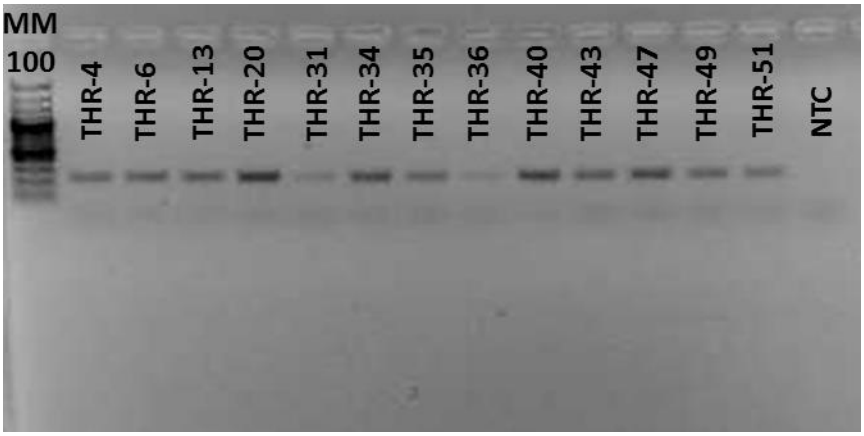
Abbreviations: SNP- Single nucleotide polymorphism, N= number of individuals, A- adenine, C- cytosine, T- Thymine, G- guanine.

Supplementary table 7-2. RNA cartilage samples and measures of NanoVue and Agilent. The samples used in gene expression are highlighted in bold.

Sample	Nanovue spectrophotometer		Agilent		
	[] ng/µl	Ratio	RIN	Ratio 28s/18s	[] ng/µl
THR-1	1580	1,838	2	0.0	1005
THR-2	37	2,028	Too low		
THR-3	112	1,488	Too low		
THR-4	**38**	**1,597**	**7.6**	**1.5**	**25**
THR-5	164	1,89	2.4	0.0	92
THR-6	**196**	**1,976**	**7.3**	**1.2**	**279**
THR-7	150	1,698	Too low		
THR-8	162	1,851	4.1	0.0	97
THR-9	51	2,084	N/A	0.0	7
THR-10	61	1,733	1.1	0.0	12
THR-11	32	1,281	N/A	0.0	3
THR-12	52	1,874	Too low		
THR-13	**55**	**1,816**	**4,2**	**1.5**	**47**
THR-14	38	1,809	N/A	0.0	5
THR-15	188	1,72	2.1	0.0	52
THR-16	162	2,283	3	0.0	50
THR-17	87	2,871	N/A	0.0	8
THR-18	38	1,646	7	0.0	37,8
THR-19	8.3	1,829	N/A	0.0	1
THR-20	**45**	**1,654**	**7.4**	**1.0**	**43**
THR-21	18	1,505	N/A	0.0	5
THR-22	42	1,957	1	0.0	14
THR-23	52	1,813	1.2	0.0	21
THR-24	16	1,718	N/A	0.0	3
THR-25	53	1,456	N/A	0.0	7
THR-26	19	1,612	3	0.0	36
THR-27	39	1,726	N/A	0.0	8
THR-28	57	1,789	N/A	0.0	5
THR-29	13	1,307	6	0.0	6
THR-30	99	1,724	2.4	0.0	31
THR-31	**59**	**1,644**	**6.5**	**0.3**	**47**
THR-32	61	1,87	6.8	0.0	12
THR-33	28	1,992	3.5	0.0	12
THR-34	**96**	**1,6**	**7.3**	**1.0**	**68**
THR-35	**331**	**1,981**	**7.5**	**1.0**	**496**
THR-36	**279**	**1,772**	**6.0**	**0.4**	**352**
THR-37	114	1,728	6.8	0.2	16
THR-38	57	1,823	6.8	0.0	14
THR-39	460	1.785	2.9	0.3	298
THR-40	**27**	**1,513**	**5.7**	**1.7**	**18**
THR-41	20	2,116	Too low		
THR-42	99	1,684	1.1	0.0	21
THR-43	**146**	**1,71**	**7.5**	**1.1**	**76**
THR-44	73	1,547	5.5	0.0	11

Sample	Nanovue spectrophotometer		Agilent		
	[] ng/µl	Ratio	RIN	Ratio 28s/18s	[] ng/µl
THR-45	54	1,476	Too low		
THR-46	42	1,907	7.3	0.0	14
THR-47	**264**	**1,976**	**7.9**	**1.1**	**401**
THR-48	15	1,393	N/A	0.0	1
THR-49	**15**	**1,851**	**7.3**	**1.0**	**14**
THR-50	264	2,091	2.8	0.0	168
THR-51	**47**	**1,987**	**7**	**0.7**	**70**
THR-52	163	1,7	Too low		
THR-53	71	11,708	2.1	0.0	40

Abbreviations: THR- Total hip replacement patient, RIN- RNA integrity number.



Supplementary figure 7-1. Conventional PCR amplification for β-actin gene (249 base pairs) on cDNA samples. Ethidium bromide stained 1.7% agarose gel. NTC- No template control, THR: Total hip replacement patient.

CHAPTER VIII:
THE ORIGIN OF *ABCC6* GENE IS POTENTIALLY LINKED WITH THE EMERGENCE OF A BONY SKELETON IN VERTEBRATES

8. THE ORIGIN OF *ABCC6* GENE IS POTENTIALLY LINKED WITH THE EMERGENCE OF A BONY SKELETON IN VERTEBRATES

8.1 Abstract

The ATP-binding cassette transporter *ABCC6* gene encodes an ABC transporter protein (MRP6) expressed primarily in the human liver and kidneys. This gene has been shown to be involved in the regulation of tissue calcification in vertebrates. We aimed to study the evolution of the *ABCC6* gene during the vertebrate radiation and characterize its potential role during the formation of the mineralized scale in the skin.

The evolution of *ABCC6* gene was performed using in silico comparative analysis and the PXE mutations were mapped in human MRP6 protein and compared with the respective amino acids positions in other species. The mRNA expression levels of *abcc6* and *abcc1* (closely related family member) were assessed during scale formation by quantitative RT-PCR on a sea bream skin/scale regeneration model.

The *abcc6* gene was only found in bony vertebrate genomes and evidence suggest that this gene was deleted from non-bony vertebrates, since no putative *abcc6* genes were retrieved from the cartilaginous fish elephant shark and lamprey. In teleosts duplication of the *abcc6* gene occurred, but the gene duplicates only persisted in the genomes of some species, suggesting that lineage or species-specific gene deletion occurred across their radiation. Nine amino acid positions, common between the fish and the mutated human MRP6 protein were found which suggest that the amino acids that cause the disease in humans, could have an important role in the calcification process in fish. No difference in *abcc6* and *abcc1* transcript abundance was detected during skin regeneration and the formation of the mineralized scale (2-3 days).

Our results provide evidence for parallel evolution of *abcc6* gene with the acquisition of a bony skeleton and highlight its potential association with calcium regulation. However, in teleost skin *abcc6* expression does not seem to be associated with scale formation/mineralization.

Keywords: *ABCC6*, *ABCC1*, *ABCC3*, Fish, PXE, vertebrates, invertebrates, MRP6.

8.2. Introduction

The ATP-binding cassette (ABC) proteins belong to a large and ancient family of active transporter proteins that are present in a broad spectrum of organisms from bacteria to vertebrates [435]. Members of this family play a pivotal role in cell physiology as they are involved in uni-or bidirectional transport [436] of a large variety of substrates in cells [435, 437]. These active transporter proteins have been exploited for cellular delivery of drugs, including cancer chemotherapeutics and thus are of major pharmacological interest in human medicine. ABC transporters are typically composed of two transmembrane domains (TMD) and two nucleotide binding domains (NBD), also known as the ATP-binding cassette [435]. The NBDs, are the motor domains of ABC transporters and are characterized by the presence of a phosphate binding loop (Walker A), a magnesium binding site (Walker B), a switch region that contains a histidine loop targeted for hydrolysis, a Q-motif that interacts with the gamma phosphate of ATP via a water molecule and a conserved signature motif (LSGCQ) that is specific of the ABC transporters [438, 439]. The NBDs together bind and hydrolyze ATP, producing the driving force for the transport of molecules across the cell membrane against a concentration gradient. The TMD participates in molecular recognition and translocation [440], and two ATP molecules are necessary for the transport of one substrate molecule across the cell membrane [441]. The human ABC gene family contains an estimated 49 members divided into seven subgroups (ABCA to ABCG), and deleterious mutations in these genes have been linked to several heritable human diseases . The ABCC (ABC subfamily C) subgroup (Table 8-1) are large proteins (1325-1582 amino acids in length) that include 8 members encoding multidrug resistance-associated proteins (MRP) and 3 members encoding ion channel proteins or regulators of potassium channels [435]. Evolutionary analysis has revealed that with the exception of the *ABCC13* gene, two major ABCC subfamily clusters exist: one that contains *ABCC1, 3, 2, 6, 8* and *9* and another that contains *ABCC4, 7, 5, 11, 12* and *10* [435]. The *ABCC6* gene encodes the multidrug resistance protein 6 (MRP6) which has recently been shown to be involved in the regulation of tissue calcification in vertebrates [122]. Calcification is the physiological mechanism by which calcium salts are deposited in body tissues. Bone formation in vertebrates is the most common example of a normal calcification process. However, calcification disorders can also occur when calcium is abnormally deposited in soft tissues causing ectopic calcification [442].

Table 8-1. Human *ABCC* genes and their functions, as listed in Ensembl and OMIM database (modified from Dean et al [400]). Thirteen genes are described and the chromosome location, number of exons and the corresponding protein are also indicated. The *ABCC6* gene is highlighted in bold as it is the research topic of the present study.

Gene	Chr location	Exons	AA	Protein	Function
ABCC1	16p13.1	31	1531	MRP1	drug-efflux pump
ABCC2	10q24.2	32	1545	MRP2	Organic anion transporter
ABCC3	17q21.33	31	1527	MRP3	Multidrug resistance
ABCC4	13q32.1	31	1325	MRP4	Nucleoside transport
ABCC5	3q27.1	30	1437	MRP5	Nucleoside transport
ABCC6	**16q13.11**	**31**	**1503**	**MRP6**	**Unknown function**
ABCC7	7q31.2	27	1480	CFTR	Chloride ion channel
ABCC8	11p15.1	39	1582	SUR1	Sulfonylurea receptor
ABCC9	12p12.1	38	1549	SUR2	Encodes the regulatory SUR2A subunit of the cardiac K+ (ATP) channel
ABCC10	6p21.1	22	1492	MRP7	Drug resistance
ABCC11	16q12.1	29	1382	MRP8	Drug resistance in breast cancer
ABCC12	16q12.1	29	1359	ABCC12	Drug resistance
ABCC13	21q11.2	Truncated protein		ABCC13	Unknown function

Abbreviations: AA- amino acid; Chr- chromosome, MRP- multidrug resistance protein, ATP- adenosine triphosphate.

The *ABCC6* gene has been associated with human calcification disorders such as human Pseudoxanthoma Elasticum (PXE; OMIM 264800), a heritable disorder characterized by calcification of elastic fibers in skin, arteries and the retina [125-127] and Generalized Arterial Calcification of Infancy (GACI; OMIM 614473), characterized by the calcification of the internal elastic lamina of muscular arteries and stenosis due to myointimal proliferation [96]. In humans, MRP6 transporter has a widespread tissue distribution and the protein is mostly detected in the basolateral membrane of hepatocytes, as well as in the proximal kidney tubules [123, 124]. Albeit with low expression, it is also found in the skin, retina and vessel walls [121, 125, 443]. Little is known about the molecules transported by MRP6 but these may be involved in stopping ectopic calcification, as in affected PXE patients MRP6 protein expression is depressed or absent [423]. MRP6 stimulates the release of adenosine triphosphate (ATP) from cells and the release of pyrophosphate (PPi) from the break-down of ATP, and is suggested to be involved in the control of calcium and other mineral deposition. Thus, the disease is not caused by the lack of functional MRP6 but rather by the absence of PPi provided to the circulation by an *ABCC6*- dependent mechanism [129].

In other vertebrates, homologues of the human *ABCC6* are also present but its role in tissue calcification is poorly understood. Teleost fish are the largest and most successful group of

extant vertebrates and their genomes have evolved and adapted during their radiation. Studies in fish have largely focused on the study of vertebrate mineralization as they share many of the basic features of the formation of cartilage (chondrogenesis) and bone (osteogenesis) tissue [111-118]. Fish, as well as mammals develop ectopic calcifications; the zebrafish *abcc6* mutant develops ectopic calcifications around perichondral bone in the craniofacial and axial skeleton [113], and zebrafish *enpp1* mutants develops ectopic calcification in a variety of soft tissues, including the skin, cartilage elements, the heart, intracranial space and the notochord sheet [114]. Zebrafish is the only teleost model system in which the *abcc6* gene has been isolated and found to be associated with tissue mineralization [111] [113]. Mutations of the zebrafish *abcc6* gene resulted in extensive hypermineralization of the axial skeleton, suggesting that functional conservation with the human exists and that it is also associated with tissue calcification in other vertebrates [113].

In order to better understand the potential association of *abcc6* genes with vertebrate calcification and mineralization we studied the evolution of the *abcc6* gene during the vertebrate radiation and characterized transcript expression during the process of scale regeneration in fish skin. In fish, scales are important skin appendages imbricated in the dermis that are a reservoir of minerals. When scales are lost, fish have the capacity to regenerate them and the formation of the new scale and scale pocket occurs immediately to protect the individual [444]. A sea bream skin regeneration model was available and it was observed that at 4 days after scale removal the skin had healed and a new scale had formed [444]. The potential involvement of *abcc6* and *abcc1* in the process of scale formation was studied by analyzing transcripts of skin and scale in the sea bream skin-scale regeneration model over 4 days of regeneration.

8.3. Methods

8.3.1. Database searches and sequence retrieval

The genomes of several vertebrates and invertebrates (with publicly available data) were explored for orthologues of the human *ABCC6* gene (ENSG00000091262) and the related family members, *ABCC1* (ENSG00000103222) and *ABCC3* (ENSG00000108846). A total of 24 vertebrate genomes representative of different classes (Supplementary table 8-1), were searched using the BLAST algorithm or database annotations for orthologues of *ABCC6*, *ABCC1* and *ABCC3* genes. The majority was deposited and assembled in the Ensembl database (GRCh38.p2) (http://ensemblgenomes.org/, accessed April 2015). The retrieved

genomes included 7 mammals (Chimpanzee, *Pan troglodytes*; Mouse, *Mus musculus*; Dog, *Canis lupus familiaris*; Armadillo, *Dasypus novemcinctus*; Cow, *Bos taurus*; Opossum, *Monodelphis domestica*; Platypus, *Ornithorhynchus anatinus*); a bird, chicken (*Gallus gallus*); the reptile green anole lizard (*Anolis carolinensis*); the amphibian Xenopus (*Xenopus tropicalis*); the Sarcopterygii Coelacanth (*Latimeria chalumnae*); 10 teleost (Actinopterigii) and 1 Agnatha (marine lamprey, *Petromyzon marinus*). The teleost genomes explored were: Tilapia, *Oreochromis niloticus;* Platyfish, *Xiphophorus maculates;* Cod, *Gadus morhua*; Stickleback, *Gasterosteus aculeatus;* Medaka, *Oryzias latipes;* Tetraodon, *Tetraodon nigroviridis;* Blind cave fish, *Astyanax mexicanus;* Zebrafish, *Danio rerio;* Spotted gar, *Lepisosteus oculatus)* and the sea bass, *Dicentrarchus labrax* assessed from the sea bass genome assembly [445]. To complement the characterization of the *ABCC* genes the genome of the elephant shark *(Callorhinchus milii*) a cartilaginous fish which diverged prior to the emergence of bony vertebrates, (Class Chondrichthyes) (http://ensembl.fugu-sg.org/index.html, accessed April 2015) was studied along with the teleost European sea bass (*Dicentrarchus labrax, http://seabass.mpipz.de/cgi-bin/hgGateway?org=European+seabass&db=dicLab1).*

To trace the likely evolutionary origin of the *ABCC6* genes the genomes of several invertebrates species were also analyzed by accessing the Ensembl Metazoa database (http://metazoa.ensembl.org/index.html, accessed April 2015). Invertebrate genomes analyzed (n = 11, supplementary table 8-2) included deuterostomes basal to the vertebrates, the tunicate Ciona (*Ciona savigny*) and the cephalochordate Amphioxus (*Branchiostoma floridae*) and the echinoderm, the Sea urchin (*Strongylocentrotus purpuratus*) and also the protostome genomes of the owl limpet (*Lottia gigantea*), the leech (*Helobdella robusta*), the fruit fly (*Drosophila melanogaster),* the Honeybee (*Apis mellifera*), the flour beetle (*Tribolium castaneum),* the malaria mosquito (*Anopheles gambiae*), the crustacean Daphnia (*Daphnia pulex*) and the nematode (*Caenorhabditis elegans).*

The amino acid sequences of putative *ABCC1*, *3* and *6* candidates were retrieved by selecting those with the lowest e-value, similarity with the query proteins and then confirming putative identity by searching against the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed April 2015) protein database using the Blastp algorithm. We also searched the sequences of the others human ABCC transporters: *ABCC2* (ENSG00000023839), *ABCC4* (ENSG00000125257), *ABCC5* (ENSG00000114770), *ABCC7* (ENSG00000001626), *ABCC8* (ENSG00000006071), *ABCC9* (ENSG00000069431), *ABCC10* (ENSG00000124574),

ABCC11 (ENSG00000121270), *ABCC12* (ENSG00000140798) and *ABCC13* (ENSG00000243064) to better resolve the origin of the vertebrate *ABCC6*, *1* and *3* members.

8.3.2. Multiple sequence alignments and sequence comparisons

The deduced amino acid sequences of *ABCC6*, *ABCC1* and *ABCC3* genes from 35 species were aligned using ClustalW (http://www.genome.jp/tools/clustalw/, accessed April 2015) and were manually edited using GeneDoc 2.7 software, to remove sequence gaps and regions of poor aligned sequences. This software was also used to calculate the percentage of sequence identity/similarity between the homologue genes of the different species. The deduced mature sequence of the full-length human *ABCC6* gene (ENSG00000091262) and the two pseudogenes *ABCC6P1* (ENSG00000256340) and *ABCC6P2* (ENSG00000255277) were also aligned using ClustalW (http://www.genome.jp/tools/clustalw/, accessed April 2015) to identify differences between the sequences.

The full-length human and vertebrate MRP6 deduced proteins were aligned using ClustalW and the amino acids that are normally changed in of the principal nucleotide mutations responsible for the manifestation of the PXE disease (http://www.hgmd.cf.ac.uk/; acceded May 2016) were mapped across the different species.

8.3.3. Phylogenetic analysis

Phylogenic trees were constructed using the deduced amino acid sequence alignment of the retrieved sequences (Supplementary table 8-1 and 8-2). We also added the sequences of the other human ABCC transporters: ABCC2, 4,5,7,8,9,10,11,12 and 13. The sequence alignment was edited in GeneDoc 2.7 software to eliminate gaps, and the edited final alignment with average sequence lengths of 1180-1200 was submitted to ProtTest 2.4 server software to select the best model to study protein evolution using the Selection Criterion of AIC (Akaike Information Criterion) [446]. The phylogenetic tree was established using the Maximum Likelihood (ML) method implemented in PhyML 3.0 software, available from the ATGC (http://www.atgc-montpellier.fr/phyml/, accessed April 2015) platform [447], using a JTT+I+G+F substitution model and the following parameters: gamma shape- 4 rate categories (G=1,109) and proportion of invariable sites (I=0.017). Sequence branch statistical support was assessed using 100 bootstrap replicates. The consensus phylogenetic tree was displayed and annotated with the FigTree4 program and was rooted using the protostome ABCC branch.

8.3.4. Gene organization and gene synteny

Human *ABCC6* and its two pseudogenes (*ABCC6P1* and *ABCC6P2*) and the *abcc6* gene from spotted gar was deduced using the Ensembl gene annotation tool. The gene structure and exon/intron sizes were compared between both species.

To characterize the gene environment of the vertebrate *ABCC6* gene we used as template the genome annotation of the Genomicus database (http://www.genomicus.biologie.ens.fr/genomicus-79.01/cgi-bin/search.pl, accessed August 2015). The human gene environment was selected, and neighboring genes were identified in the following vertebrates (Opossum *(Monodelphis domestica)*, Platypus *(Ornithorhynchus anatinus)*, Chicken (*Gallus gallus)*, Xenopus *(Xenopus tropicalis)*, Coelacanth *(Latimeria chalumnae)*, Spotted gar (*Lepisosteus oculatus)*, Zebrafish (*Danio rerio*), Tetraodon (*Tetraodon nigroviridis*), Stickleback *(Gasterosteus aculeatus)*, Medaka (*Oryzias latipes*) and the sea bass (*Dicentrarchus labrax*) using genome annotations available from the genome assemblies. The neighboring genes were characterized and identified based upon Ensembl database gene annotation and complemented with sequence homology searches (http://www.ensembl.org/Multi/Tools/Blast?db=core, accessed May 2015). The Elephant shark (*Callorhinchus milii)* homologue genome region was also characterized using a similar strategy to that described above. The accession numbers used for linkage analysis are available in supplementary table 8-3.

8.3.5 Transcriptome database searches

To increase knowledge about the physiological importance of the *ABCC* genes, searches on vertebrate transcriptome data were also performed. The “in silico” distribution of the non-mammalian *ABCC6* was characterized and the transcriptome and EST (Expressed sequence tag) databases of the human, bird, reptile, amphibian and teleost were searched to construct a digital expression map to infer possible overlapping tissue expression of the *ABCC6* gene with human. Searches were performed in lineage-specific NCBI EST databases human (taxid: 9606); birds (taxid:8782); reptiles (taxid:8504); amphibians (taxid:8292) and teleost fishes (taxid:32443) using the human, chicken, anole lizard, xenopus and zebrafish *abcc6* sequences, respectively. The identity of the sequences retrieved was confirmed by homology with the human orthologues. Searches were also performed in other databases where expression data has been deposited: Geisha (http://geisha.arizona.edu/geisha/), Xenbase (http://www.xenbase.org/entry/), Expression Atlas Database

(https://www.ebi.ac.uk/gxa/home), GeneCards (http://www.genecards.org/), Ensembl (http://www.ensembl.org/index.html) and complemented with published data [113].
In addition, a sea bass scale and skin transcriptomes available "in house" was also searched for *abcc6* and *abcc1* transcripts (Pinto et al., unpublished).

8.3.6 Polymerase chain reaction (PCR) and quantitative-PCR (qPCR)

Based on the sea bass transcriptome data we further explored the potential physiological role of the abcc transcripts in fish skin and scale formation. *abcc6* and *abcc1* genes were studied in the skin/scale of sea bream (*Sparus auratus*) using a regenerating skin model over a 4 days period as previously described [444]. The experiments were carried out following international and national guidelines for animal care and experimentation, under a "Group-I" licence from the Portuguese Government Central Veterinary service to CCMAR and conducted by a certified investigator (DMP).
Briefly, adult sea bream of the same age class (1 year) were used and at time zero scales were removed from the left flank of the body by gently stroking the skin with forceps to minimize damage of the dermis. Samples (N = 8/ time point) of intact skin (untouched right hand flank) and damaged skin (left hand flank) were collected during a time series after scale removal: 6 h (disruption of the dermis and scale pocket) and 24 h (reorganization of the epidermis to wound heal), 48h (emergence of dermal papilla that defines the localization of the new scale), 72h (scale emergence) and 96h (scale mineralization). In this way, the same fish provided control and regenerating skin samples and they could be directly compared. Specific primers for the sea bream *abcc6* and *abcc1* were designed by searching the CCMAR sea bream transcriptome database with transcripts from various tissues [448]. The primers for *abcc6* were Sb_abcc6fwd ttagagacaagacccgcat and Sb_abcc6rev tggcaaaggtgtggatgaag and for *abcc1* were Sb_*abcc1fwd* tatgagtcacctcaacaaagc and Sb_*abcc1rev* tccgttcatactggattacca. Preliminary expression analysis of *abcc6* and *abcc1* by PCR on sea bream cDNA (bone, skin, larva, scale) (available in the laboratory) was carried out using the following cycle: 95ºC, 3 min; (95ºC 30 sec, 60ºC 30 sec, 72ºC 30 sec) cycled 40 times and a final extension of 72ºC, 5 min. The amplified PCR products were sequenced to confirm their identity before testing for gene expression on the skin regeneration model.
To investigate the potential involvement of *abcc6* and *abcc1* in sea bream skin regeneration a preliminary qPCR experiment was run using cDNA pools composed of RNA from both intact and damaged skin in 6 individuals for each time point. The qPCR was performed for a 10µl final reactions volume using the 1× SsoFast-Evagreen Supermix (Biorad) and 300 nM of

forward and reverse primers. Reactions were performed using the StepOnePlus thermocycler (Applied Biosystems, UK) following the program: 30 s at 95 °C, 45 cycles of 5 s at 95 °C and 15 s at 60 °C. A final melting curve was carried out between 60 and 95 °C and produced a single product dissociation curve for each gene. Relative expression between the damaged and intact skin samples were compared using the delta Ct values normalized with the housekeeping gene *rps18*.

8.4. Results

8.4.1. Members of the *ABCC6* in vertebrates and invertebrates

Orthologues of the human *abcc6* gene were identified in all the teleost and tetrapod genomes analyzed with the exception of two fishes: the cartilaginous fish elephant shark (*Callorhinchus milii*) and the lamprey (*Petromyzon marinus*). In some teleost species such as the stickleback (*Gasterosteus aculeatus*), blind cave fish (*Astyanax mexicanus*) and zebrafish (*Danio rerio*) more than one *abcc6* gene was identified. In stickleback and blind cave fish two *abcc6* genes were found and in the zebrafish three seem to exist (Figure 8-1).

Multiple sequence alignment of the deduced amino acid sequence of the *ABCC6* gene sequences revealed a relatively high conservation overall and several highly conserved regions (alignment of *ABCC6* and *ABCC1* genes of vertebrates available in supplementary figure 8.1). Comparison of the deduced mature protein sequence of the full-length MRP6 transcripts in human with the orthologue in the chicken (*Gallus gallus)* indicated that the deduced proteins shared 52% amino acid identity and 69% similarity. Between the human and the lobe finned fish Coelacanth (*Latimeria chalumnae*) the MRP6 proteins shared 48% identity and 67% similarity and 42-47% identity and 61-65% similarity with the orthologues in the teleosts, stickleback *(Gasterosteus aculeatus), European sea bass (Dicentrarchus labrax),* tetraodon *(Tetraodon nigroviridis),* tilapia *(Oreochromis niloticus), m*edaka *(Oryzias latipes),* platyfish, *(Xiphophorus maculates),* cod (*Gadus morhua),zebrafish (Danio rerio),* blind cave fish *(Astyanax mexicanus)* and spotted gar (*Lepisosteus oculatus)* (Supplementary table 8-4). *ABCC6*-related family members, the *ABCC1* and *ABCC3* genes, were also retrieved from the databases for evolutionary comparisons because, a) they are similar to *ABCC6* and b) *ABCC1* in the human genome is localized in proximity to the *ABCC6* locus.

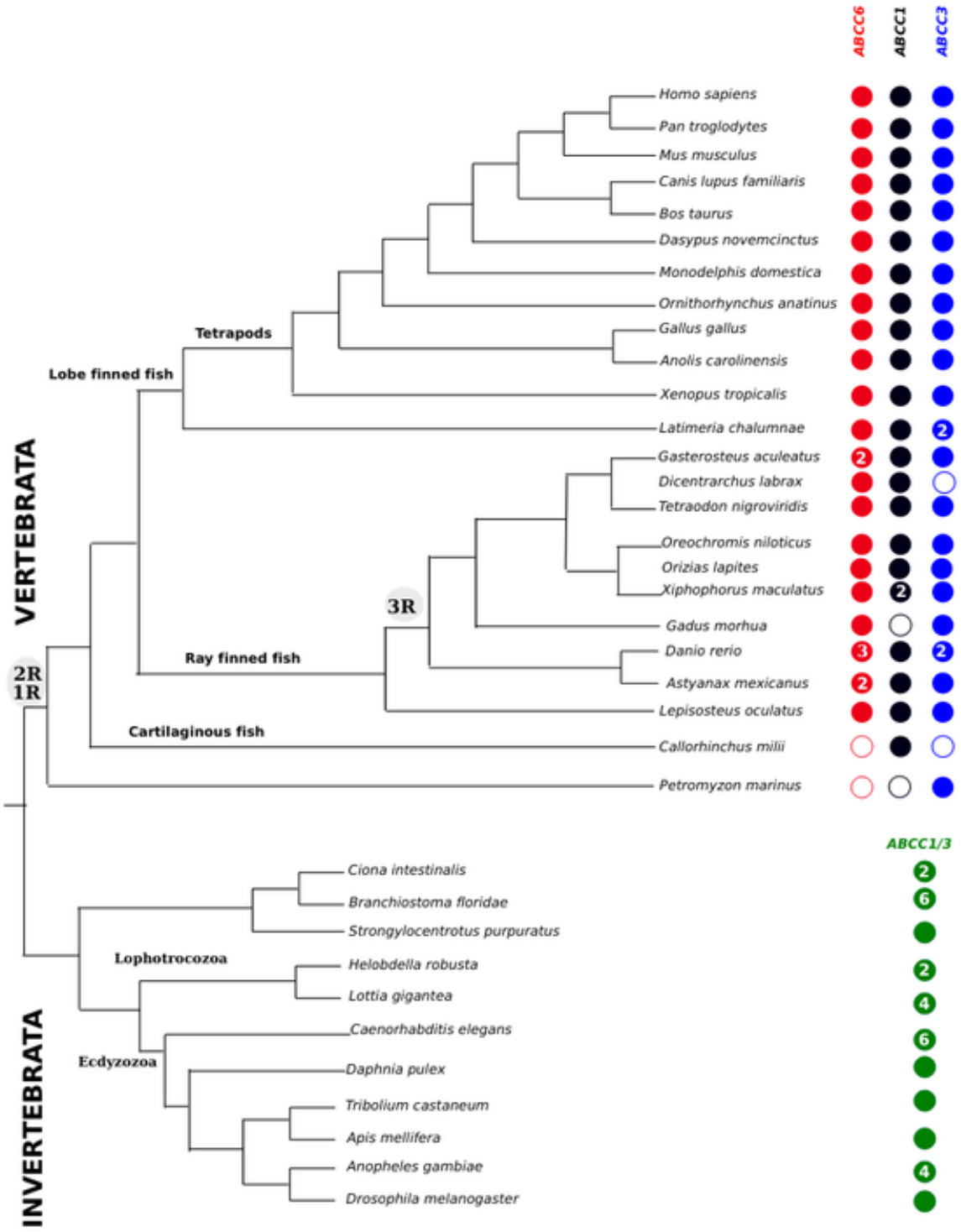
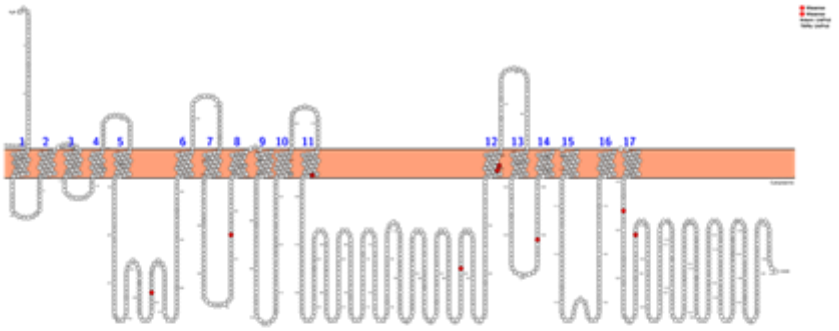


Figure 8-1. Dendrogram indicating the number of *ABCC6* genes and the related *ABCC1* and *ABCC3* genes identified in the different vertebrate and invertebrate genomes. Circles with different colours represent members of the different gene families and uncoloured circles means the gene was not identified. The dendrogram was drawn having in consideration the evolutionary relationship of the different species and the two major genome duplication events (1R and 2R) that are proposed to have occurred early in the vertebrate radiation. The teleost specific genome duplication event (3R) is also indicated. Gene accession numbers are available in supplementary table 8-1 and 8-2.

Orthologues of the human *ABCC1* and *ABCC3* genes were also identified in almost all vertebrates with the exception of *abcc1* in cod (*Gadus morhua*) and lamprey (*Petromyzon marinus*) genomes, and *abcc3* in the sea bass (*Dicentrarchus labrax)* and the cartilaginous fish elephant shark (*Callorhinchus milii*) (Figure 8-1). It is unclear if the absence of the *abcc1* and *abcc3* genes in several species is the result of their elimination from these species genomes or if they are the consequence of incomplete genome assemblies. Teleost duplicate genes were also found for *abcc1* in platyfish (*Xiphophorus maculatus*) and for *abcc3* in coelacanth (*Latimeria chalumnae*) and zebrafish (*Danio rerio*) (Figure 8-1). Searches in early deuterostomes and protostome genomes were also carried out to characterize the origin of the *abcc6, 1 and 3* genes. Queries using the human MRP6 sequence identified putative *abcc6*-like genes in several invertebrate genomes and many species seem to possess multiple gene copies (Supplementary table 8-2).

8.4.2 Sequence conservation of the amino acids altered in PXE disease

A total of 139 mutation associated with PXE disease were retrieved from Human Gene Mutation database (HGMD) and the amino acids positions altered by mutation were mapped in the alignment and compared across the different vertebrate species. Mutations have been identified throughout the protein however the “hot spots” associated with PXE is more located within the cytoplasmatic region of human MRP6. Comparisons of the vertebrate homologue amino acid positions revealed that in general the mutations mapped in the alignment are well conserved across mammals (Supplementary figure 8-1). More than 50 amino acid positions are 100% conserved across vertebrates (Supplementary figure 8-1), suggesting that these positions were under high selective conservative pressure during the vertebrate radiation. Interestingly, in some species, especially within fish, there are positions in which the amino acid in several vertebrates corresponds to the mutated amino acid in human MRP6. The amino acid positions, common between the fish and the mutated human MRP6 protein, map to the cytoplasmatic region (252; 415; 881; 1049; 1226 and 1268) and the transmembrane region (594; 946 and 950) and are represented in Figure 8-2.



Protein region	CTD	CTD	TMD	CTD	TMD		CTD	CTD	
AA position	232	415	594	881	946	950	1049	1226	1268
Codon change in human	CTT-TTT	GTG-GCG	GCC-GTC	AGG-AGT	CTC-ATC	GCA-ACA;GTA	TCC-GCC	CTA-ATA	CGG-CAG
Human variation in PXE disease	F	A	V	S	I	T;V	A	I	Q
Human	L	V	A	R	L	A	S	L	R
Chimpanzee	L	V	A	R	L	A	S	L	R
Mouse	L	V	A	G	L	T	S	L	R
Cow	L	V	A	G	L	A	S	L	R
Dog	L	V	A	E	L	A	S	L	R
Armadillo	L	V	A	G	L	A	S	L	R
Opossum	L	V	A	T	I	V	S	L	R
Platypus	L	V	A	K	G	T	S	L	R
Chicken	A	V	A	R	L	I	S	I	R
Anolis lizard	F	V	A	Q	A	I	S	I	R
Xenopus	F	T	S	P	Y	S	A	M	Q
Coelacanth	L	V	A	T	L	V	S	I	R
Stickleback_a	F	A	A	S	I	I	S	V	E
Stickleback_b	L	A	A	S	I	I	S	V	Q
Robalo	L	A	A	S	I	I	A	V	Q
Tetraodon	L	A	A	E	I	I	A	V	Q
Platyfish	L	A	A	S	I	I	A	V	Q
Cod	-	A	X	S	L	I	A	V	Q
Zebrafish_a	L	A	A	S	L	I	A	V	Q
Zebrafish_b	L	A	V	S	F	I	S	V	Q
Zebrafish_c	L	A	A	H	L	I	S	V	Q
Blind cave fish_a	L	A	A	S	L	I	S	V	Q
Blind cave fish_b	L	A	A	S	L	I	A	V	Q
Medaka	L	A	A	S	I	I	V	V	Q
Spotted gar	L	A	A	S	L	I	S	V	R

TMD: Transmembrane domain
CTD: Citoplasmatic domain
EXT: Extracellular

Figure 8-2. Alignment of the MRP6 amino acid positions affected by 16 selected mutations previously associated with PXE disease in human and in other vertebrates in which amino acid that cause PXE in humans is the normal in other species.

Comparisons of *ABCC6* and *ABCC1* revealed that the *ABCC6* PXE point mutation are also generally highly conserved across this related family member, suggesting that they may also play a pivotal role in the maintenance of ABCC family function.

8.4.3 Phylogeny of *ABCC6*

Phylogenetic analysis was performed using the protein alignment of the deduced vertebrate MRP6, MRP1 and MRP3 mature proteins and their putative homologues in invertebrates (early deuterostomes and protostomes). The tree topology shows that *ABCC6* and the two

other members are in three independent branches (with strong bootstrap support) and that the family members shared common ancestry prior to the vertebrate emergence (Figure 8-3). The tree suggests that *ABCC3* members diverged earlier than *ABCC1* and *ABCC6* genes that seem to be the result of a more recent gene duplication. The invertebrate branch contains several ABCC-like genes that apparently share a common ancestor with the vertebrate genes but they form a separate clade and do not group with any of the vertebrate protein clusters. Several duplications of genes occurred in invertebrate species and also in some vertebrates, such as the teleosts. Based upon the sequence clustering in the tree, gene duplication of *abcc6* seems to be the result of the teleost specific whole genome duplication event and in the zebrafish subsequent species-specific gene duplications also occurred.

The tree topology, failed to cluster the coelacanth sequences ENSLACG00000022117 (annotated as *abcc1* in ensembl database) and ENSLACG00000001471 (annotated as *abcc6* in ensembl database) in the expected branch. According to the clustering in the phylogenetic tree the sequence ENSLACG00000022117 is an *abcc6* gene, and the sequence ENSLACG00000001471 is an *abcc1* gene. The absence of the *abcc6* gene in lamprey (*Petromyzon marinus)* and cartilaginous fish elephant shark (*Callorhinchus milli)* suggests that *abcc6* was potentially deleted in early vertebrates. The Xenopus *(Xenopus tropicalis) abcc6* gene does not group as expected based on the consensus in relation to species evolution, suggesting that abcc6 in this specie suffered distinct selective pressures or there may be an errors in the genome or incomplete genome assemblies. The Coelacanth (*Latimeria chalumnae*) *abcc3* gene did not group as expected since the putative coelacanth *abcc3* sequence (ENSLACG00000006619) was very incomplete.

Addition of the other human ABCC transporters revealed that the early deuterostomes sequences identified shared common ancestry with vertebrate *ABCC6*, *1* and *3* members and that the protostome members retrieved from the insect and nematode genomes seem to have shared a common origin with the *ABCC2* family early in the ancestral bilaterial genome (Figure 8-3).

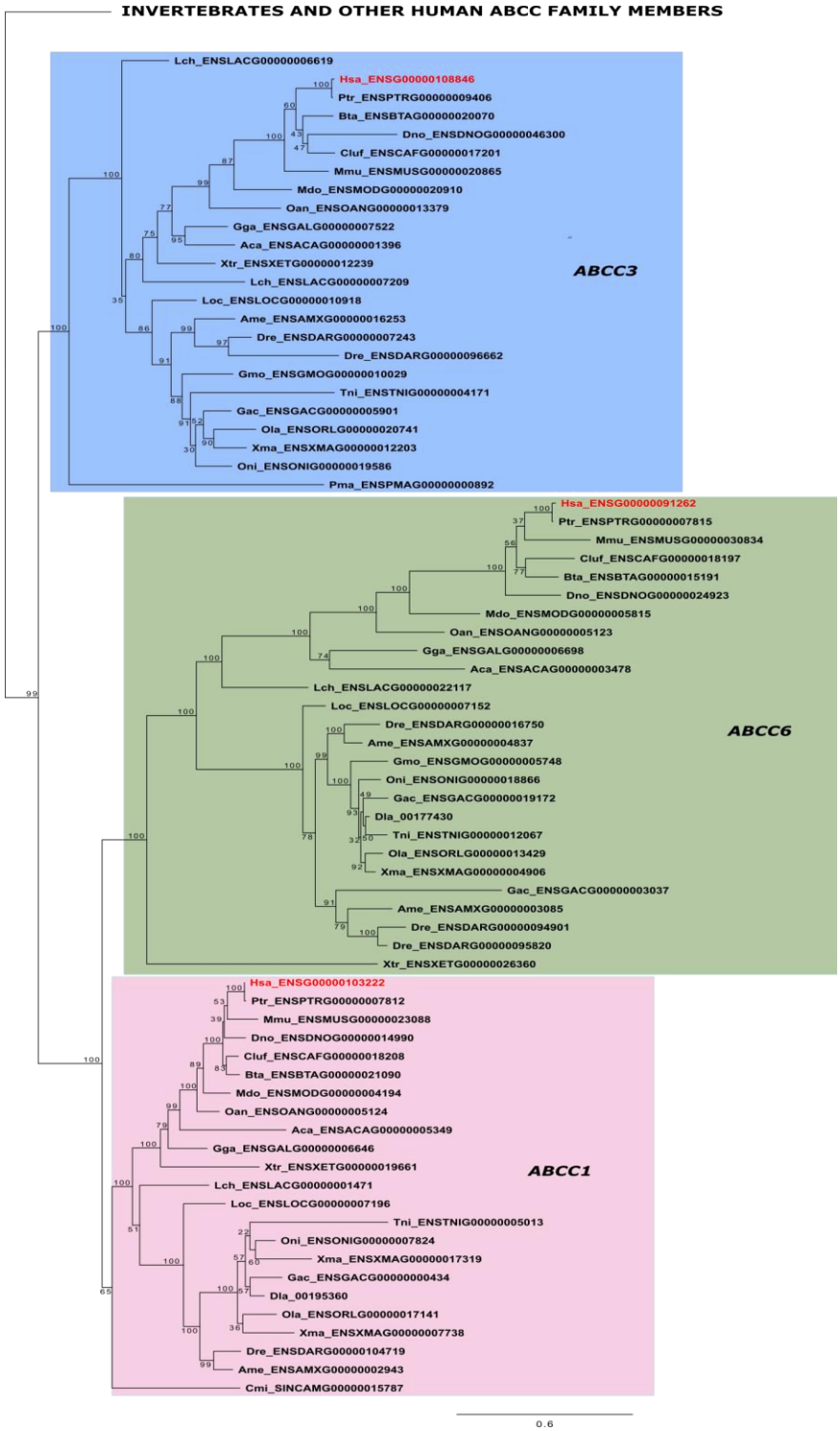


Figure 8-3. Phylogenetic tree of the vertebrate and invertebrate ABCC6, 1 and 3 with the other human ABCC family members. The phylogenetic tree was built using the ML method with PhyML 3.0 software with 100 bootstrap replicates using JTT+I+G+F substitution model given by Protest and was rooted using the invertebrate ABCC-like branch. To facilitate interpretation, the three major vertebrate ABCC clades are boxed with different colours, and the detailed presentation of the invertebrates and the other human ABCC family members (ABCC2, 4, 5, 7, 8, 9, 10, 11 and 12) have been collapsed. The original tree is available in supplementary figure 8-2 and the accession numbers are given in the supplementary tables 8-1 and 8-2.

8.4.4. Gene structure and gene linkage across vertebrate

The gene structure of the human *ABCC6* genes (including the two *ABCC6* pseudogenes *ABCC6P1* and *ABCC6P2*) was compared with the fish spotted gar *abcc6* gene (Figure 8-4). In human, the full *ABCC6* gene structure is composed of 31 exons and spans 74,56kb in chromosome 16. The two pseudogenes are much smaller and map in close proximity to the full-length *ABCC6* gene. *ABCC6P1* is composed of 11 exons and *ABCC6P2* is composed of 5 exons which span 27,14Kb and 3,88 Kb, respectively (Figure 8-4). Exons 2, 3, 4 and 5 of *ABCC6P2*, *ABCC6P1* and *ABCC6* have almost the same sequence and only differ at exon 1. Exon 8, 9 and 10 of *ABCC6P1* have the same coding potential as *ABCC6* and the differences in the remaining exons may be a consequence of their divergence after gene duplication.

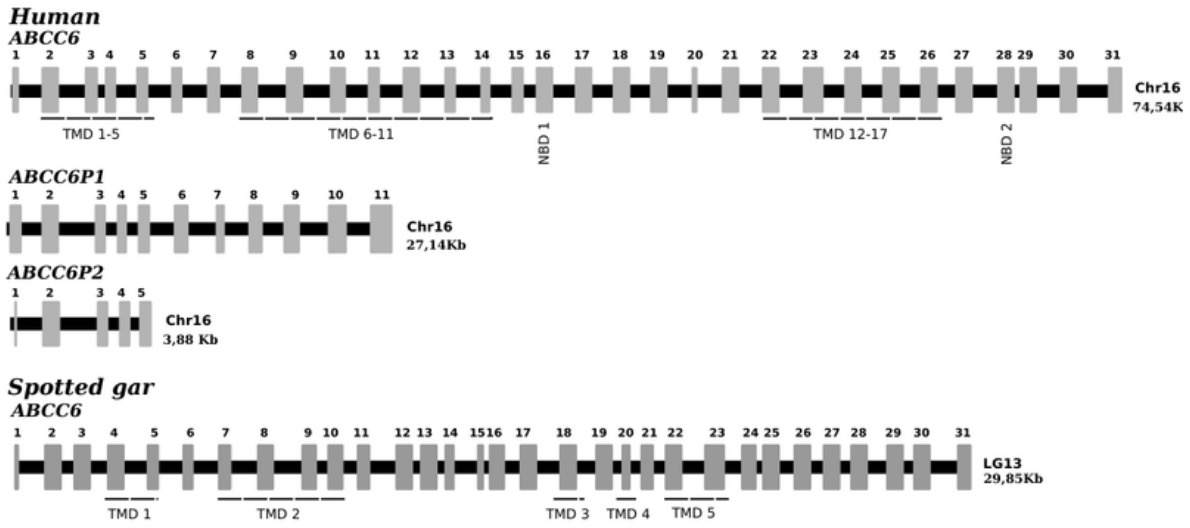


Figure 8-4. Gene organization of the *ABCC6* gene in human and spotted gar. The two incomplete human *ABCC6* pseudogenes (*ABCC6P1* and *ABCC6P2*) are also represented. Gene sizes are indicated and exons are represented by numbered boxes and the solid black line denotes the introns.

No putative *ABCC6* pseudogenes were found in other vertebrate genomes; they seem to be characteristics of the human genome. Comparison of the predicted *in silico* gene structure of the human and spotted gar (a slow evolving teleost genome, that has not suffered tetraploidization, 3R) revealed that they share the same exon number and most exons (21 exactly) are of similar size (Supplementary table 8-5). Exon 15 and Exon 16 are the most divergent and they encode a region of low conservation within the cytoplasmic motif.

Several conserved genes flank the vertebrate *ABCC6* gene suggesting that evolution of this chromosome segment in vertebrates was under conservative pressures. The genes *ABCC1*; *COQ7*; *ARL6IP1*; *RPS15A*; *NOMO3*; *XYLT1*; *MYH11*; *FOPNL* and *NDE1* were found in the

vicinity of the *ABCC6* gene across many vertebrate genomes. Gene synteny analysis revealed that with the exception of the *abcc6* gene in the Elephant shark (*Callorhinchus milli)* and *ARL6IP1* in Platypus (*Ornithorhynchus anatinus)* and *FOPNL* in Coelacanth (*Latimeria chalumnae)* and Xenopus (*Xenopus tropicalis)* at least ten genes that are part of the human *ABCC6* gene environment have been maintained across vertebrates (Figure 8-5). In the genomes of primates a conserved gene environment with the human and other vertebrates was also identified but no putative *ABCC6* pseudogenes were found further suggesting that these were acquired during the radiation of the human.

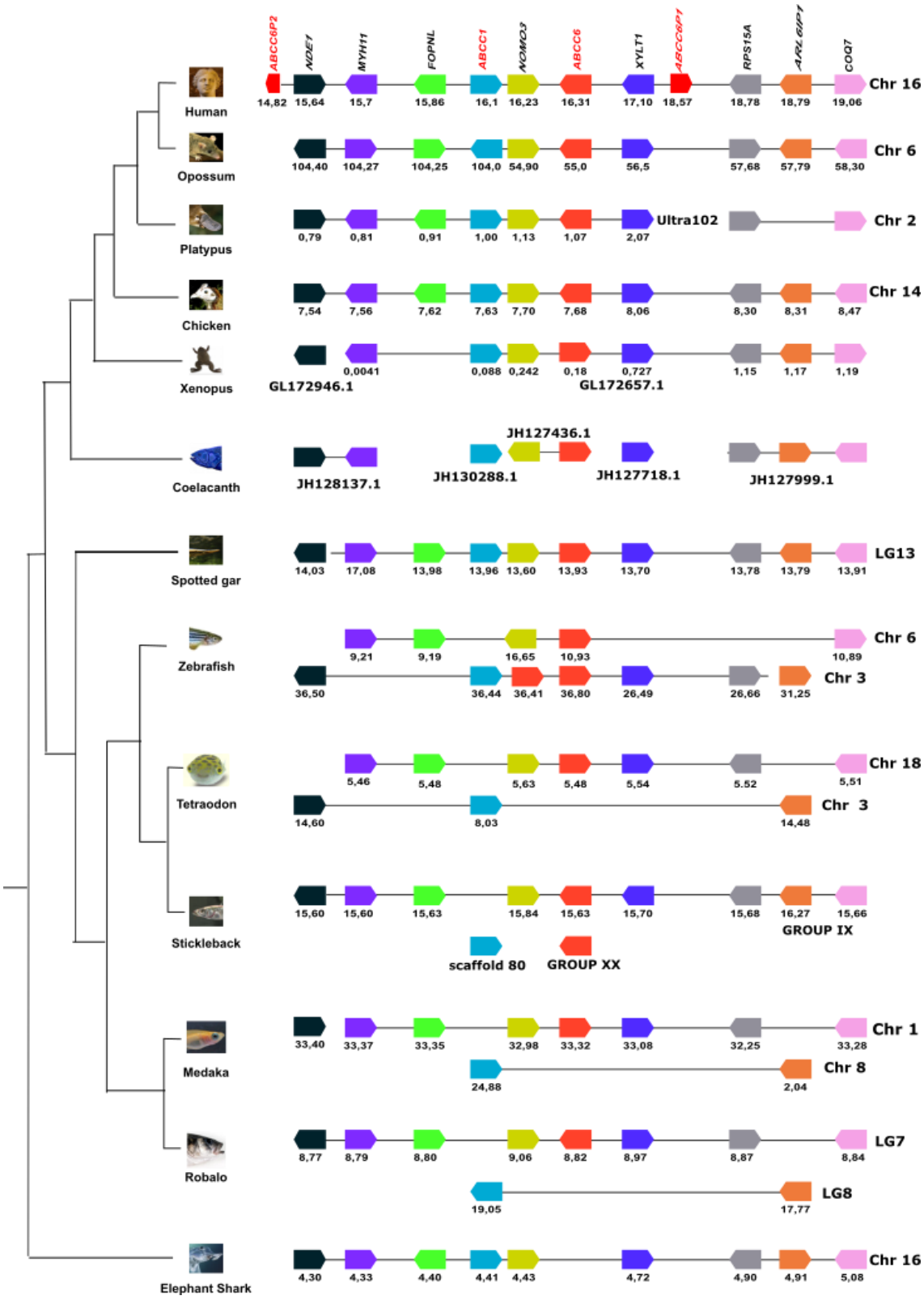


Figure 8-5. Gene synteny analysis of the *ABCC6* gene environment across vertebrates. The Human, Opossum, Platypus, Chicken, Xenopus, Coelacanth, Zebrafish, Stickleback, Spotted gar and Elephant shark homologue genome regions are represented. Arrowheads represent gene orientation in the genome. Members of the same family are indicated by the same color. The position of the genes in the genome fragments is indicated.

8.4.5 Expression analysis in non-mammals

Searches on ESTs and several other databases for non-mammalian vertebrates revealed the tissue distribution of the *ABCC6* transcripts (Table 8-2). A similar strategy was also applied for the sequence homologues *ABCC1* and *ABCC3* for comparisons. As in humans, expression of these transcripts seems to be widespread in different tissues including some with importance in calcium homeostasis. In the chicken, *ABCC6* was found to be expressed in epiphyseal growth plate but also in the intestine and kidney, which are important organs that regulate calcium balance in vertebrates [442]. In reptiles, an EST was found in the kidney and in teleost fish *abcc6* transcripts were found in the craniofacial bone elements, fins and intervertebral discs. *ABCC1* and *ABCC3* seem to have a more dispersed distribution than *ABCC6* (Table 8-2).

Table 8-2. *ABCC6*; *ABCC1* and *ABCC3* expression analysis in human, bird, reptile, amphibian and teleost. ESTs were obtained from NCBI EST databases. Searches were also performed in other databases: Geisha (http://geisha.arizona.edu/geisha/); Xenbase (http://www.xenbase.org/entry/); Expression Atlas (https://www.ebi.ac.uk/gxa/home), GeneCards (http://www.genecards.org/), Ensembl http://www.ensembl.org/index.html) and complemented with published data [113].

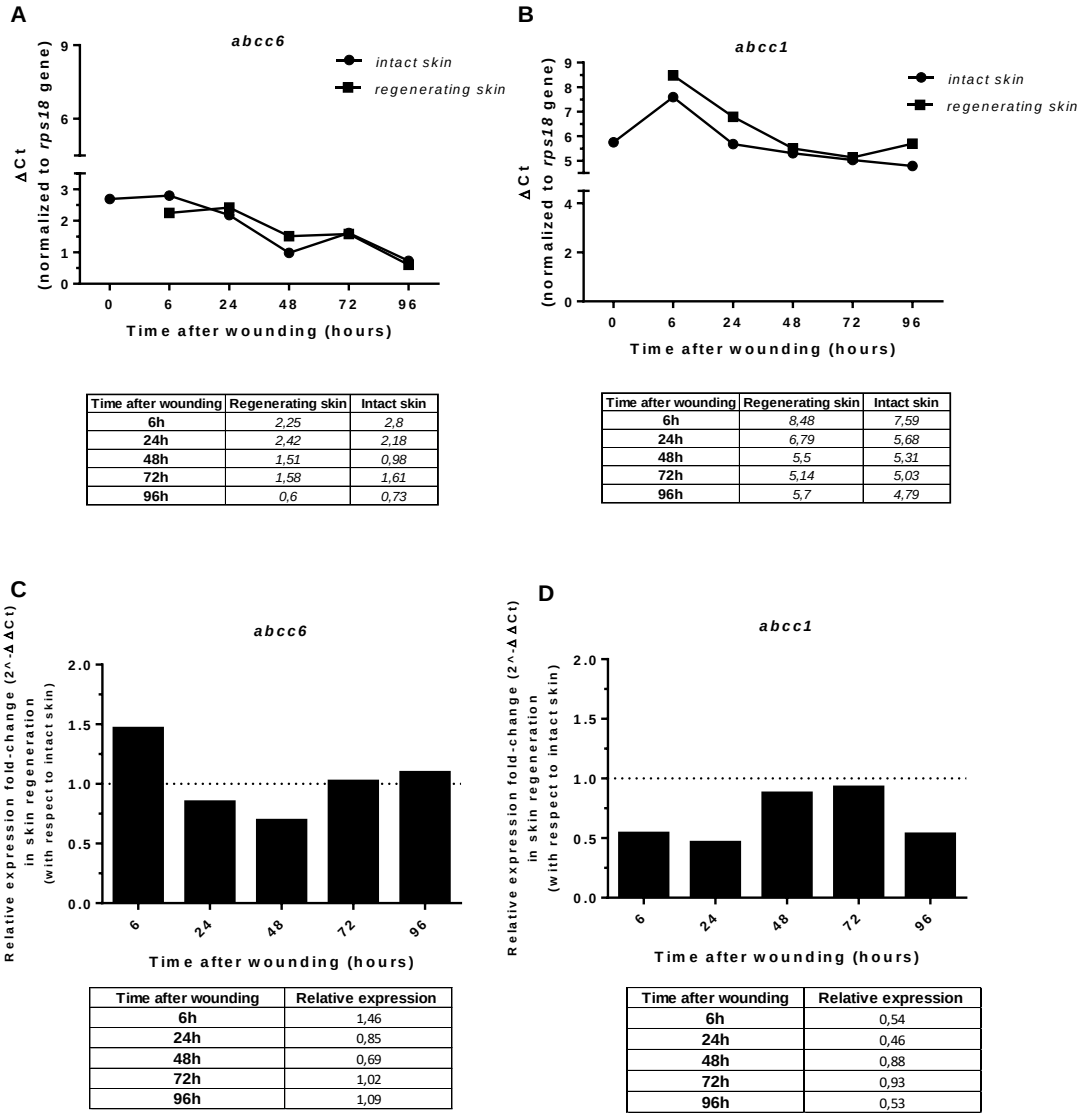
Tissue	*ABCC6*				*ABCC1*				*ABCC3*			
	Hsa	Gga	Aca	Dre	Hsa	Gga	Xtr	Dre	Hsa	Gga	Aca	Xtr
Adenocarcinoma cell line									X			
Adrenal gland					X				X			
Aorta					X							
Ascites					X							
Blood						X						
Brain					X	X						
Bursa of fabricius						X						
Bursal lymphocyte						X						
Carcinoma, cell line									X			
Cerebrum	X											
Cervix, tumor tissue									X			
Chondrocytes isolated from growth plate cartilage						X						
Chondrosarcoma lung metastasis cell lines					X				X			
Colon									X			
Connective tissue						X						
Cortex	X											
Craniofacial bone elements				X								
Day 1 embryo								X				
Ductal carcinoma, cell line									X			
Dura mater					X							
EBV-transformed					X							
Embryonic stem cells, cell lines					X							
Embryonic tissue						X						
Epididymis					X							
Epiphyseal growth plate		X				X						
Epithelioid carcinoma					X							
Esophageal, tumor tissue									X			

Tissue	*ABCC6*				*ABCC1*				*ABCC3*			
	Hsa	**Gga**	**Aca**	**Dre**	**Hsa**	**Gga**	**Xtr**	**Dre**	**Hsa**	**Gga**	**Aca**	**Xtr**
Esophagus muscle					**X**							
Fins				**X**								
Gallbladder	**X**											
Gastroesophage					**X**							
Gonad		**X**				**X**						
Gonadal PGC					**X**	**X**						
Head (Geisha)		**X**				**X**						
Heart						**X**	**X**				**X**	**X**
Hepatocelular carcinoma, cell line	**X**											
Human embryonic stem cells					**X**							
Intestine							**X**					
Invertebral disc				**X**								
Kidney	**X**	**X**	**X**				**X**			**X**	**X**	**X**
Large cell carcinoma									**X**			
Large intestine	**X**											
Leukemia cell					**X**							
Leukocyte									**X**			
Liver	**X**	**X**				**X**						
Liver and Spleen									**X**			
Lung					**X**							
Lymph node					**X**							
Mitral valve					**X**							
Neuroblastoma					**X**				**X**			
Ovary		**X**			**X**	**X**			**X**			
Pancreas	**X**								**X**			
Paratiroid gland					**X**							
Peripheral Nervous system									**X**			
Pooled colon, kidney, stomach									**X**			
Pterygium									**X**			
Renal cell adenocarcinoma									**X**			
Retinoblastoma					**X**							
Skeletal muscle						**X**	**X**					
Skin					**X**			**X**				
Small intestine		**X**										
Smooth muscle					**X**							
Spinal cord	**X**											
Spleen					**X**	**X**			**X**			
Splenocytes						**X**						
Stomach					**X**				**X**			
Testis					**X**			**X**				
Thalamus									**X**			
Thymus					**X**				**X**			
Thyroid					X							
Tongue					X							
Tongue, tumor tissue					X				X			
Trunks		X										
Uterus					X							
Vas deferens					X							
Whole embryo						X	X	X				
Whole larva								X				
Whole body								X				

Abbreviations: Hsa- Human, Gga- Chicken, Aca- Anole lizard, Dre- Zebrafish, Xtr- Xenopus.

8.4.6 Expression of abcc6 and abcc1 during sea bream scale formation

According to the CT values (Supplementary table 8-6), both transcripts for *abcc6* and *abcc1* are expressed in the sea bream skin and, *abcc6* seems to be more expressed than *abcc1* (Supplementary table 8-6). Comparison of the Ct values between the regenerating and intact skin samples suggests that gene expression in both samples is similar and follows a similar trend, and there was no evidence for major changes in cycle number, indicating that *abcc6* and *abcc1* genes not have a role in skin regeneration and scale formation in the teleost. The same results were observed in figure 8-6A and 8-6B in which both intact and regenerating skin samples, the genes shows also a similar distribution profile.



Time after wounding	Regenerating skin	Intact skin
6h	2,25	2,8
24h	2,42	2,18
48h	1,51	0,98
72h	1,58	1,61
96h	0,6	0,73

Time after wounding	Regenerating skin	Intact skin
6h	8,48	7,59
24h	6,79	5,68
48h	5,5	5,31
72h	5,14	5,03
96h	5,7	4,79

Time after wounding	Relative expression
6h	1,46
24h	0,85
48h	0,69
72h	1,02
96h	1,09

Time after wounding	Relative expression
6h	0,54
24h	0,46
48h	0,88
72h	0,93
96h	0,53

Figure 8-6. Expression results of *abcc6* and *abcc1* in sea bream skin regeneration assay. A and B shows the profile of ΔCT values in *abcc6* and *abcc1*; ΔCT values were obtained subtracting the Ct of each gene by the housekeeping control gene *rps18*. C and D represent the relative expression of *abcc6* and *abcc1* genes in regenerating skin relative to intact skin.

Comparisons of the relative expression levels between regenerating (ΔΔCt method) and intact sea bream skin (Figure 8-6C and 8-6D) revealed that expression of *abcc6* and *abcc1* between regenerating and intact skin was similar.

8.5. Discussion

In the present study the evolution of the vertebrate *ABCC6* gene was studied and the potential role of this gene in fish skin scale regeneration was tested. Orthologues of the human *ABCC6* gene were explored in several vertebrate genomes and phylogenetic analysis revealed that human and other vertebrate *ABCC6* genes shared common ancestry and that the *abcc6* gene emerged early during the vertebrate radiation. The *abcc6* gene was only found in bony vertebrates genomes and evidence suggests that this gene was deleted from non-bony vertebrate, as no putative *abcc6* genes were retrieved from the cartilaginous fish elephant shark and the lamprey. The teleost duplication of the *abcc6* gene occurred but the gene duplicates only persisted in the genomes of some species, suggesting that lineage or species-specific gene deletion occurred during their radiation. In the mapping of the human PXE mutations we obtained nine amino acid positions, common between the fish and the mutated human MRP6 protein, located in cytoplasmatic and transmembrane regions. This could suggest that the amino acids that cause the disease in humans, may have an important role in the calcification process in fish. Expression analysis of *abcc6* gene during the mineralization process in scales in the sea bream regeneration model suggested that this gene may not be involved in scale formation, even though it was highly expressed in fish skin. Our results provide evidence for parallel evolution of the *abcc6* gene with the acquisition of a bony skeleton and highlights its potential association with calcium regulation, however the physiological role of the ABCC gene family in teleost skin remains to be determined.

Orthologues of the human *ABCC6* were found in the genomes of different vertebrates. The non-mammalian genes that shared high sequence and structure similarities with the human *ABCC6* gene were retrieved from birds and fish. In tetrapods and in the majority of teleosts and also in the coelacanth and spotted gar, a single *abcc6* gene was found, however in the stickleback and blind cave fish two paralogue genes persisted and in the zebrafish genome three were identified. During the vertebrate radiation, duplication and deletion events have greatly contributed to shape vertebrate genome content [449]. Early as the vertebrates emerged two whole round of genome tetraploidization occurred prior to the divergence of the jawless fish [450, 451]. In the teleosts, a subsequent genome duplication event occurred and this explains the presence of gene duplicates in teleost genomes relative to tetrapods [451].

However, only some duplicates persisted and other were eliminated from the genome [452] potentially due functional redundancy [453]. The reason why duplicated *abcc6* genes persisted in stickleback, blind cave fish and zebrafish and were deleted from the genomes of other analyzed teleost is unknown, and it remains to be established if both gene copies are functional.

Although functional studies of the *abcc6* genes in teleosts are scarce, in zebrafish some studies exist [111-113]. The zebrafish skeleton shows high similarity with human bones in terms of cells, matrix proteins, and molecular signaling pathways [454]. A study revealed that, in zebrafish embryos, the duplicate *abcc6a* is expressed in the kupffer's vesicles and tail bud, while the second duplicate - *abcc6b*- is expressed in the enveloping layer and embryonic kidney proximal straight tubules [111]. In the same study, using morpholinos to "knock-down" *abcc6a* and *abcc6b* the authors suggested that *abcc6a* is an essential gene for normal fish development [111].

It is known that the absence of the MRP6 activity observed in patients with PXE or *Abcc6*-/- observed in mice, cause ectopic mineralization [128, 415]. A study using zebrafish mutants revealed that disease causing *abcc6* mutations, that do not directly affect the transport and or ATP catalytic activity, resulted primarily in lower stability and or cytoplasmatic retention of the mutant proteins [112]. Mackay and Schulte-Merker identified, by next generating sequencing, the causative mutation in the *abcc6a* gene in a zebrafish line with a recessive mineralization disorder. The identified mutation resulted in the substitution of L1429R in a highly conserved region of NBD-2 that contains the Walker B motif, which is essential for binding to ATP [455]. In another study, the authors verified that in zebrafish this variant causes hypermineralisation of the axial skeleton, resulting in mineralised structures in the intervertebral space. According to Ensembl database, this variant could also occur in the cow (L1426R; rs440576475), but no phenotype information was available. In humans, in the corresponding variant (L1425P; rs150230403) a proline substitution occurs instead of an arginine, and is not associated with PXE or any other disease. However the Sift and PolyPhen rating suggest that this variant has a deleterious and damaging effect on the protein. The immediately preceding variant I1424T in humans is associated with PXE.

In our study, we show that *abcc6* and the related *abcc1* are expressed in teleost skin and we hypothesized that it could be involved in scale formation. However expression studies in a sea bream skin/scale regeneration model suggests that the gene expression varies across time but they were not significantly different from the control, and thus the potential role of *abcc6* in skin remains undefined. In zebrafish the *abcc6a* gene is expressed and functions at the site of

mineralization, secreting ATP from cells increasing PPi locally, suggesting that the transporter ligand in fish is not liver derived as occurs in humans [113].

A strong link between the *ABCC6* gene and reduced amounts of pyrophosphate has been established [129]. *ABCC6* overexpression induces nucleotide release in vitro, which is rapidly converted by ENPP1 into PPi [129]. It has been reported that PPi secretion from the livers of *Abcc6*-/- mice was dramatically lower compared to wild type mice, and the authors suggested that MRP6 is an ATP efflux transporter [397]. ATP is converted into AMP and PPi and represents the main source of mineralization inhibitor PPi in plasma, which fully explains why the absence of *ABCC6* results in the ectopic mineralization observed in patients with PXE [397].

Our preliminary expression analysis in a teleost skin regeneration mode revealed that the *abcc6* gene may not be involved in early stages of scale formation. In fact it should be noted that mineralization in PXE and its mouse model *Abcc6*-/-, is not noted at birth, but develops later in life. Probably the *abcc6* gene in fish skin acts as a potent inhibitor of pathological ectopic calcification through a PPi mechanism. Overall, the presence of the *abcc6* gene in teleost fishes and its absence in cartilaginous and lamprey genomes suggest that this gene possibly emerged associated with the need for more sophisticated mechanisms of control of PPi in cells. Future studies aimed at understand the function of *abcc6* in teleost skin will be required.

8.6 Supplementary Material

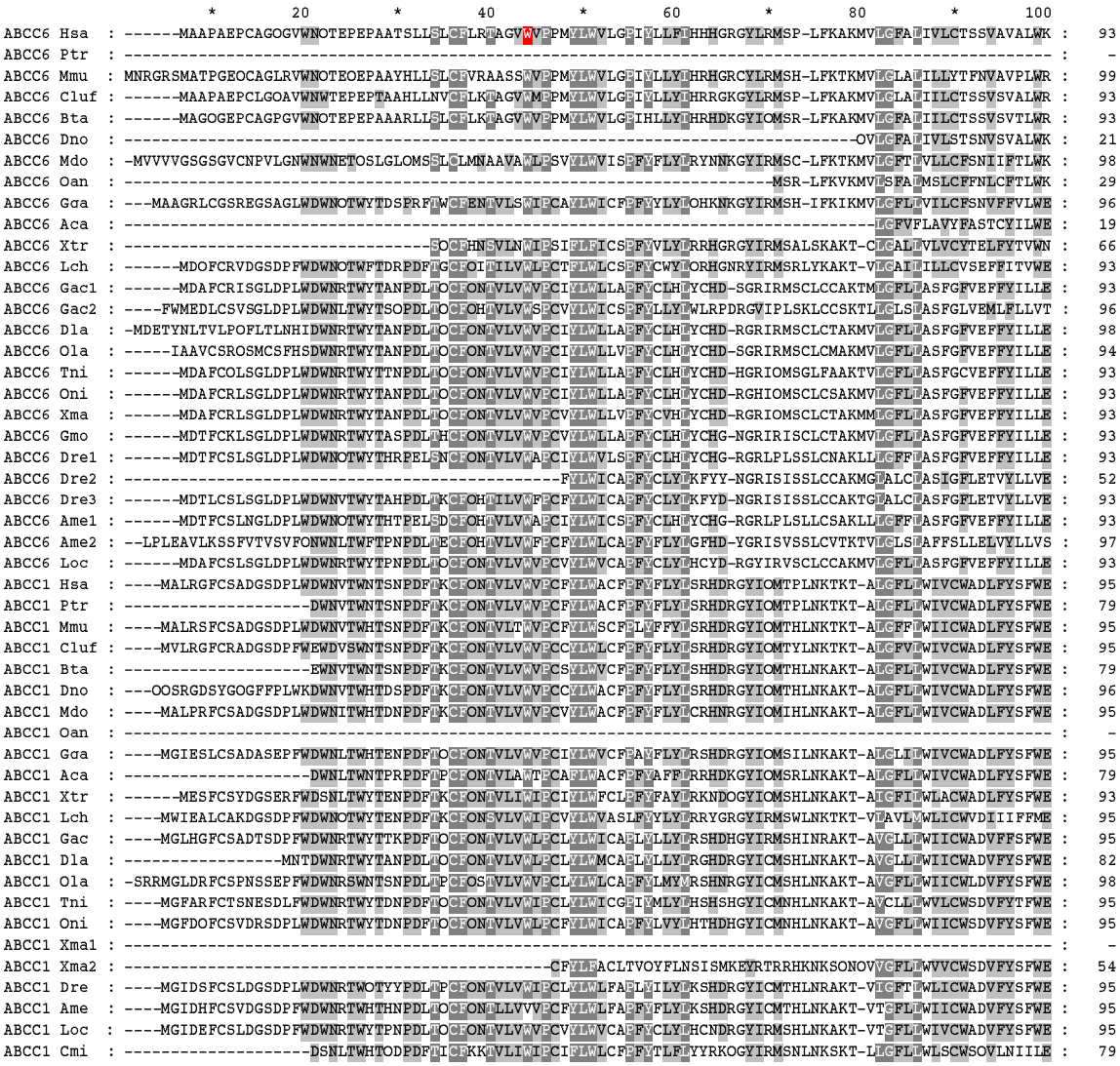
Supplementary table 8-1. Accession numbers of the *ABCC6*, *ABCC1* and *ABCC3* genes retrieved from vertebrates. Sequences were retrieved from Ensembl Genomes (http://ensemblgenomes.org/, accessed April 2015) database. The Elephant shark *(Callorhinchus milii*) orthologues were obtained from (http://ensembl.fugu-sg.org/index.html, accessed April 2015) database.

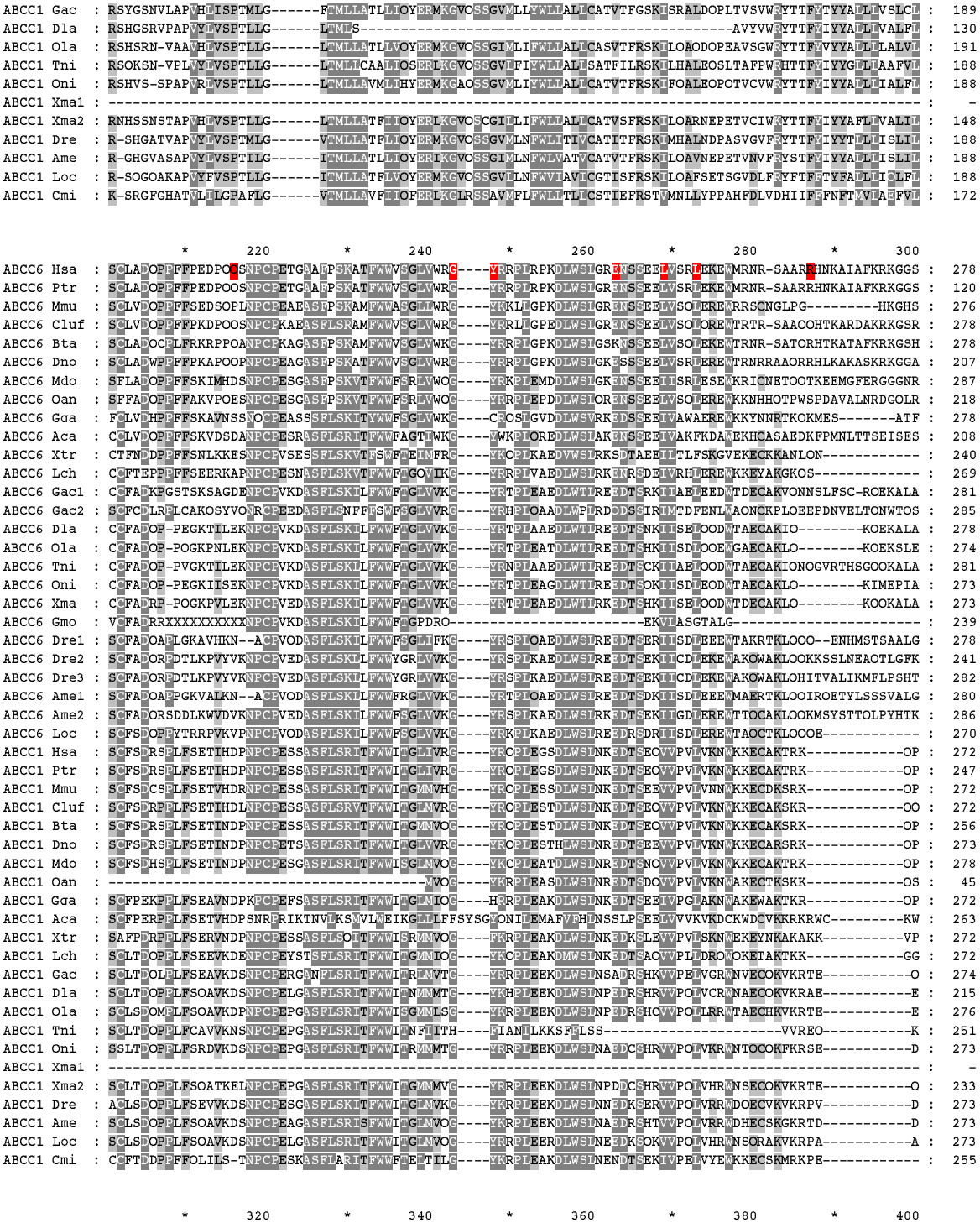
Vertebrates					
Specie name	**Common name**	**Abbreviatures**	**Accession number**		
			ABCC6	***ABCC1***	***ABCC3***
Homo sapiens	Human	Hsa	ENSG00000091262	ENSG00000103222	ENSG00000108846
Pan troglodytes	Chimpanzee	Ptr	ENSPTRG00000007815	ENSPTRG00000007812	ENSPTRG00000009406
Mus musculus	Mouse	Mmu	ENSMUSG00000030834	ENSMUSG00000023088	ENSMUSG00000020865
Canis lupus familiaris	Dog	Cluf	ENSCAFG00000018197	ENSCAFG00000018208	ENSCAFG00000017201
Bos taurus	Cow	Bta	ENSBTAG00000015191	ENSBTAG00000021090	ENSBTAG00000020070
Dasypus novemcinctus	Armadillo	Dno	ENSDNOG00000024923	ENSDNOG00000014990	ENSDNOG00000046300
Monodelphis domestica	Opossum	Mdo	ENSMODG00000005815	ENSMODG00000004194	ENSMODG00000020910
Ornithorhynchus anatinus	Platypus	Oan	ENSOANG00000005123	ENSOANG00000005124	ENSOANG00000013379
Gallus gallus	Chicken	Gga	ENSGALG00000006698	ENSGALG00000006646	ENSGALG00000007522
Anolis carolinensis	Anole Lizard	Aca	ENSACAG00000003478	ENSACAG00000005349	ENSACAG00000001396
Xenopus tropicalis	Xenopus	Xtr	ENSXETG00000026360	ENSXETG00000019661	ENSXETG00000012239
Latimeria chalumnae	Coelacanth	Lch	ENSLACG00000022117	ENSLACG00000001471	ENSLACG000000007209 ENSLACG00000006619
Gasterosteus aculeatus	Stickleback	Gac	1) ENSGACG00000019172 2) ENSGACG00000003037	ENSGACG00000000434	ENSGACG00000005901
Dicentrarchus labrax	See bass	Dla	177430	195360	ni
Oryzias latipes	Medaka	Ola	ENSORLG00000013429	ENSORLG00000017141	ENSORLG00000020741
Tetraodon nigroviridis	Tetraodon	Tni	ENSTNIG00000012067	ENSTNIG00000005013	ENSTNIG00000004171
Oreochromis niloticus	Tilapia	Oni	ENSONIG00000018866	ENSONIG00000007824	ENSONIG00000019586
Xiphophorus maculatus	Platyfish	Xma	ENSXMAG00000004906	1) ENSXMAG00000017319 2) ENSXMAG00000007738	ENSXMAG00000012203
Gadus morhua	Cod	Gmo	ENSGMOG00000005748	ni	ENSGMG00000010029
Danio rerio	Zebrafish	Dre	1) ENSDARG00000016750 2) ENSDARG00000094901 3) ENSDARG00000095820	ENSDARG00000104719	1) ENSDARG00000007243 2) ENSDARG00000096662
Astyanax mexicanus	Blind cave fish	Ame	1) ENSAMXG00000004837 2) ENSAMXG00000003085	ENSAMXG00000002943	ENSAMXG00000016253
Lepisosteus oculatus	Spotted gar	Loc	ENSLOCG00000007152	ENSLOCG00000007196	ENSLOCG00000010918
Callorhinchus milii	Elephant shark	Cmi	ni	SINCAMG00000015787	ni
Petromyzon marinus	Lamprey	Pma	ni	ni	ENSPMAG00000000892

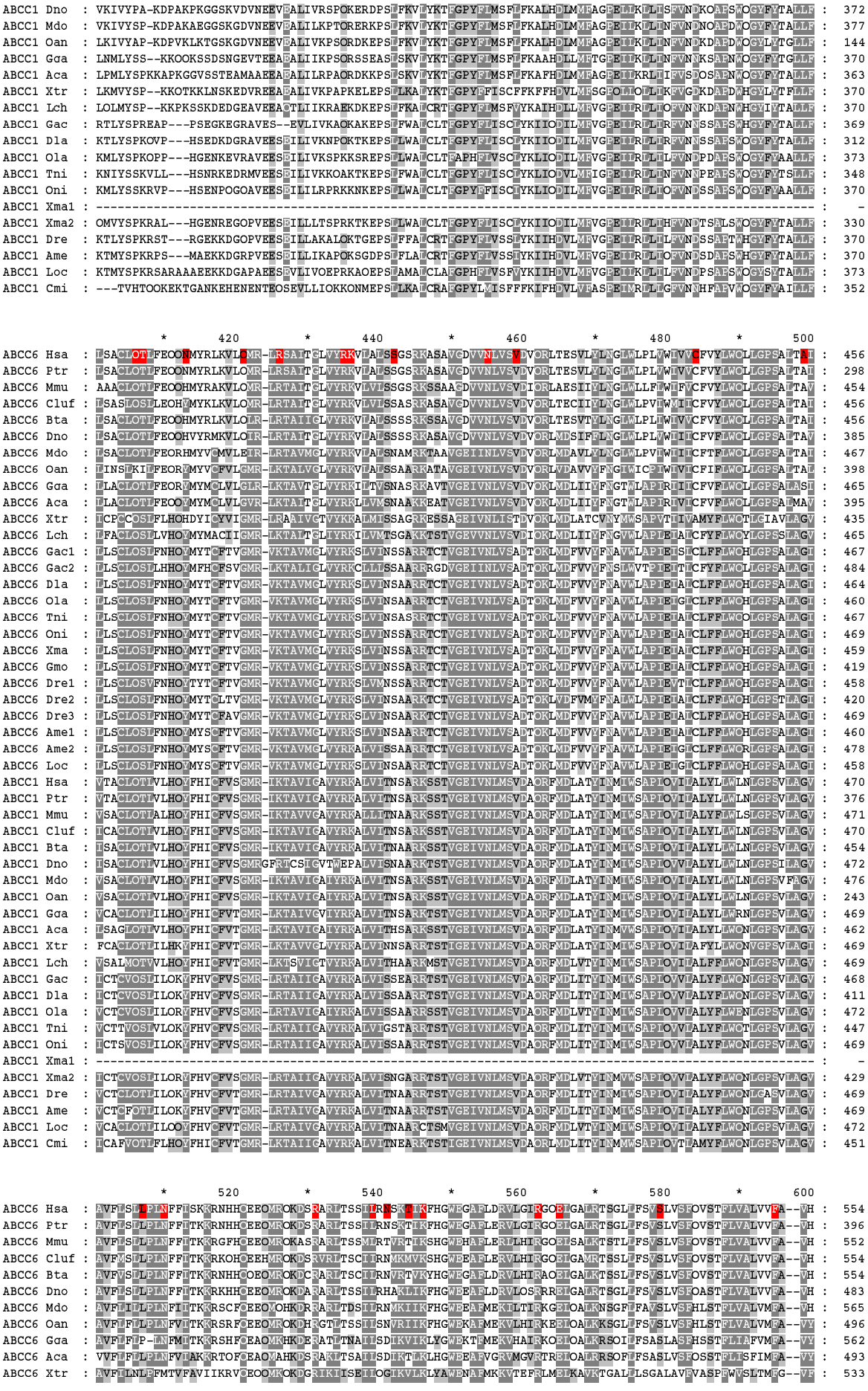
Abbreviations: ni- not identified

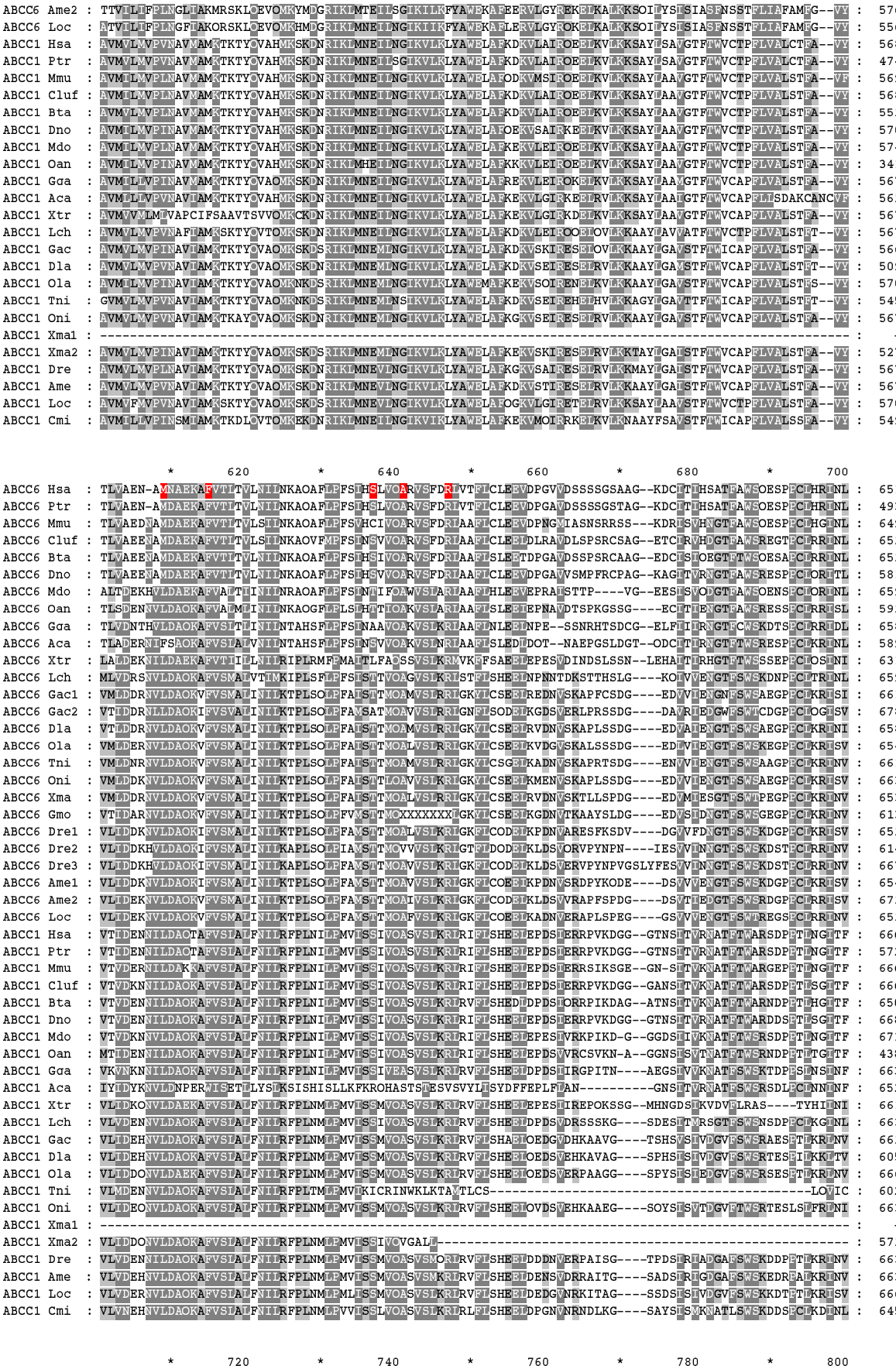
Supplementary table 8-2. Accession numbers of the *ABCC* genes retrieved from invertebrates. Sequences were retrieved from Ensembl Metazoa database (http://metazoa.ensembl.org/index.html, accessed April 2015).

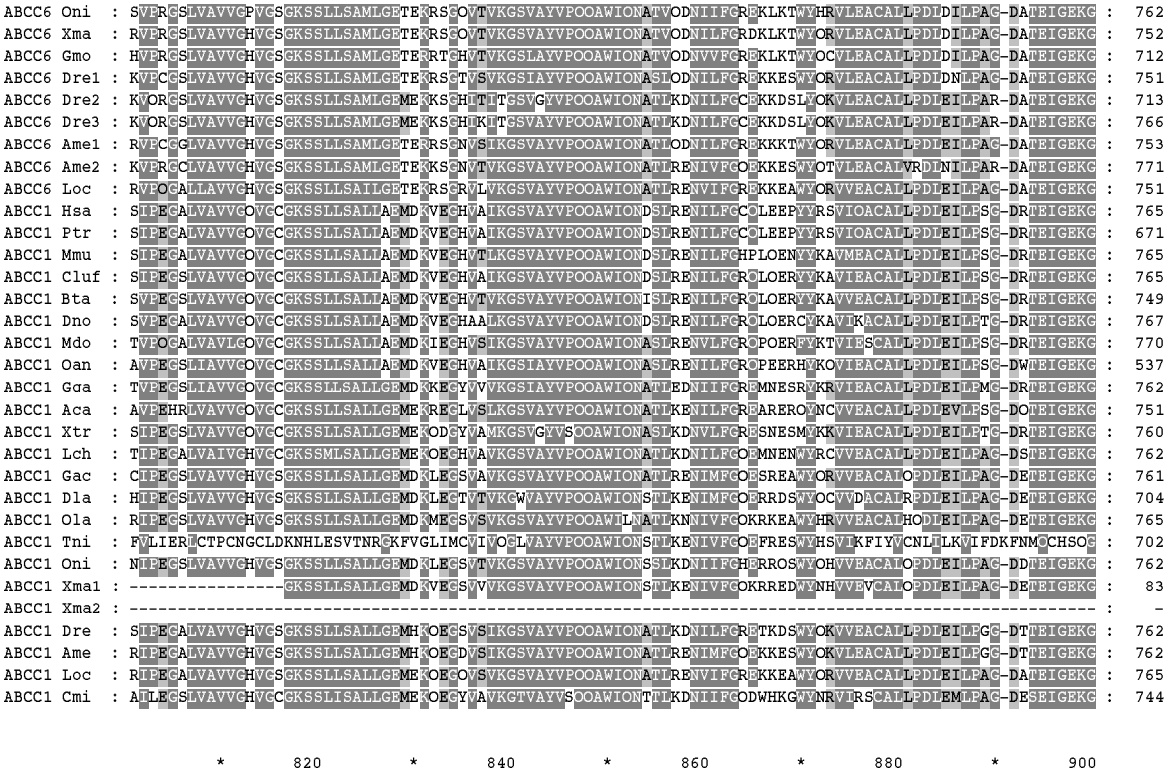
Invertebrates			
Specie name	**Common name**	**Abbreviation**	**Accession number**
			abcc1/3/6
Caenorhabditis elegans	Roundworm	Cel	WBGene00003407
			WBGene00003408
			WBGene00003410
			WBGene00003413
			WBGene00003409
			WBGene00003414
Daphnia pulex	Water flea	Dpu	DAPPUDRAFT_347281
Anopheles gambiae	Malaria mosquito	Aga	AGAP009835
			AGAP008437
			AGAP027980
			AGAP028128
Tribolium castaneum	Red flour beetle	Tca	TC012253
Apis mellifera	Honeybee	Amel	GB53134
Drosophila melanogaster	Drosophila	Dme	FBgn0032456
Helobdella robusta	Leech	Hro	HelroG163344
			HelroG157076
Lottia gigantea	Owl limpet	Lgi	LotgiG107213
			LotgiG105097
			LotgiG110718
			LotgiG153611
Strongylocentrotus purpuratus	Sea urchin	Spu	26395
Ciona intestinalis	Ciona	Csa	ENSCSAVG00000003792
			ENSCSAVG00000008135
Branchiostoma floridae	Anphioxus	Bfl	118638
			232174
			118636
			128060
			90918
			230771

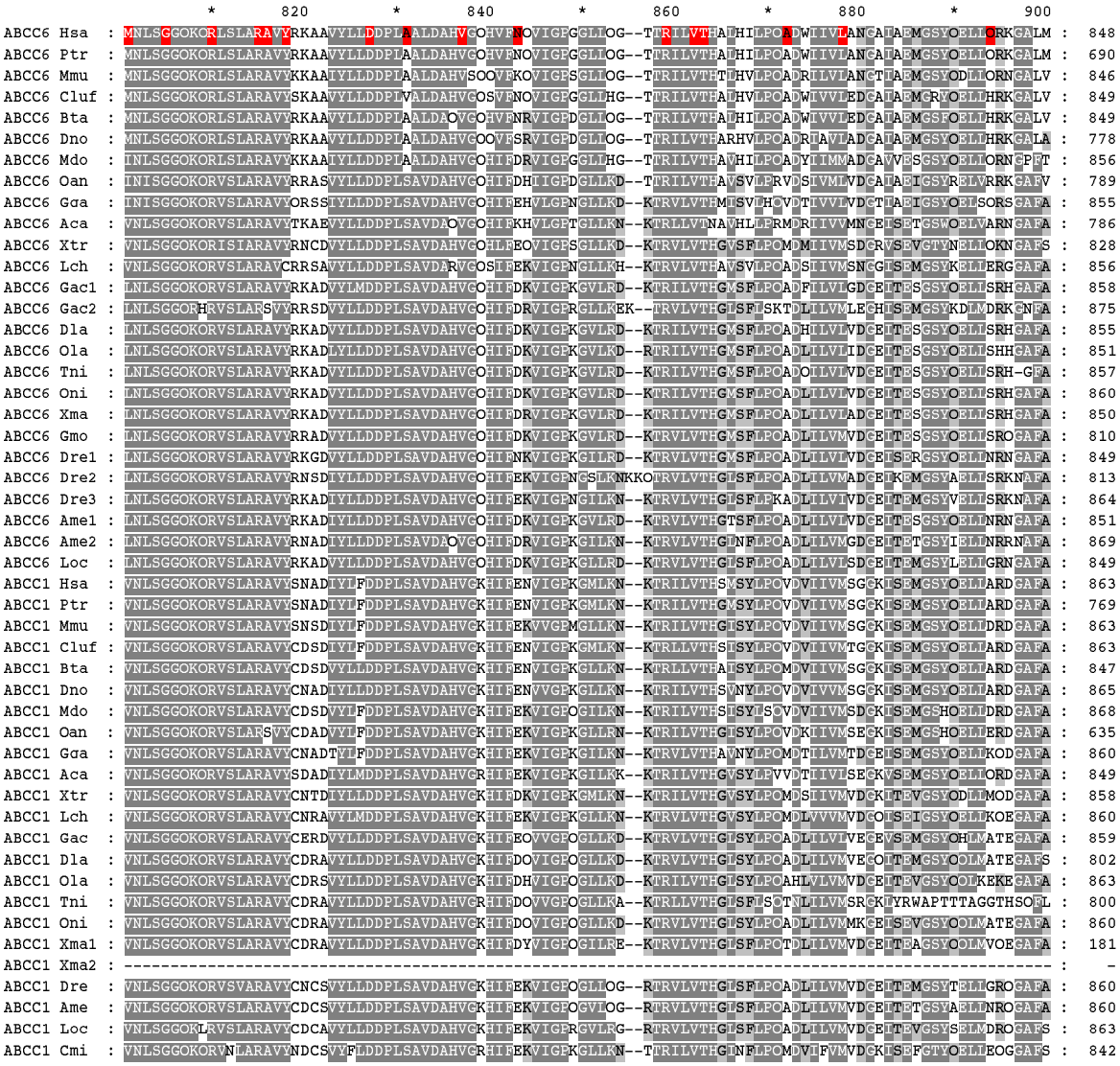


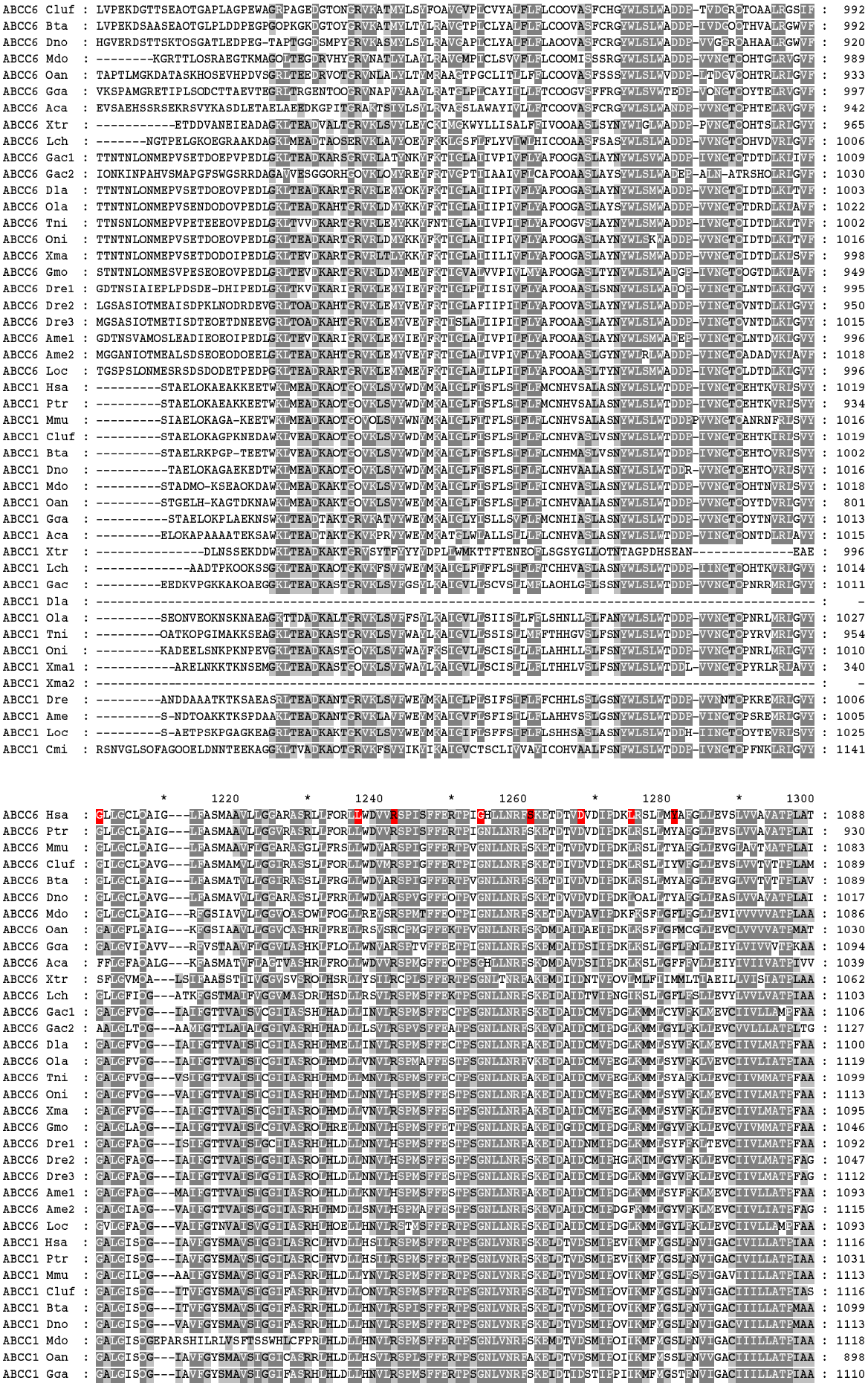


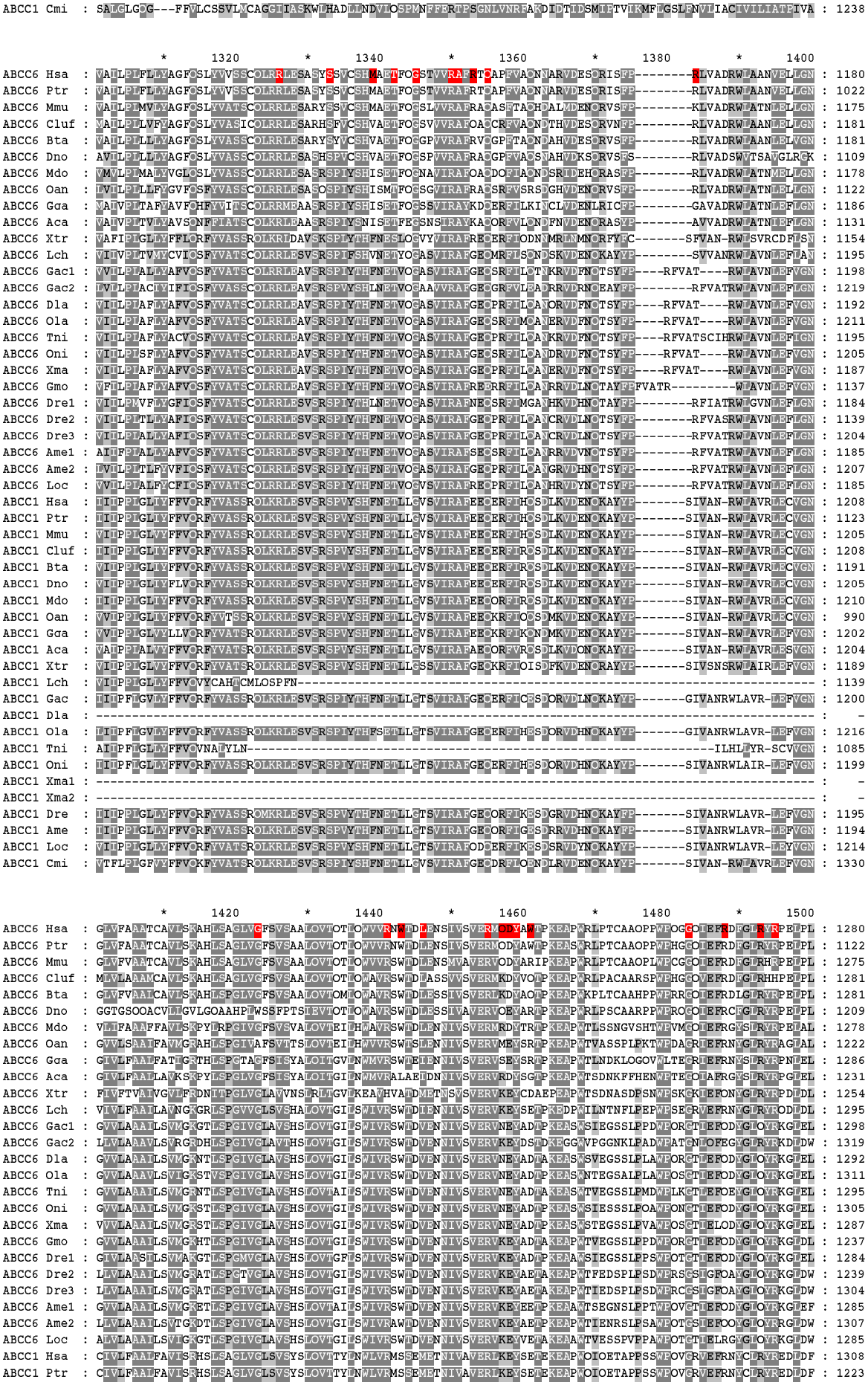


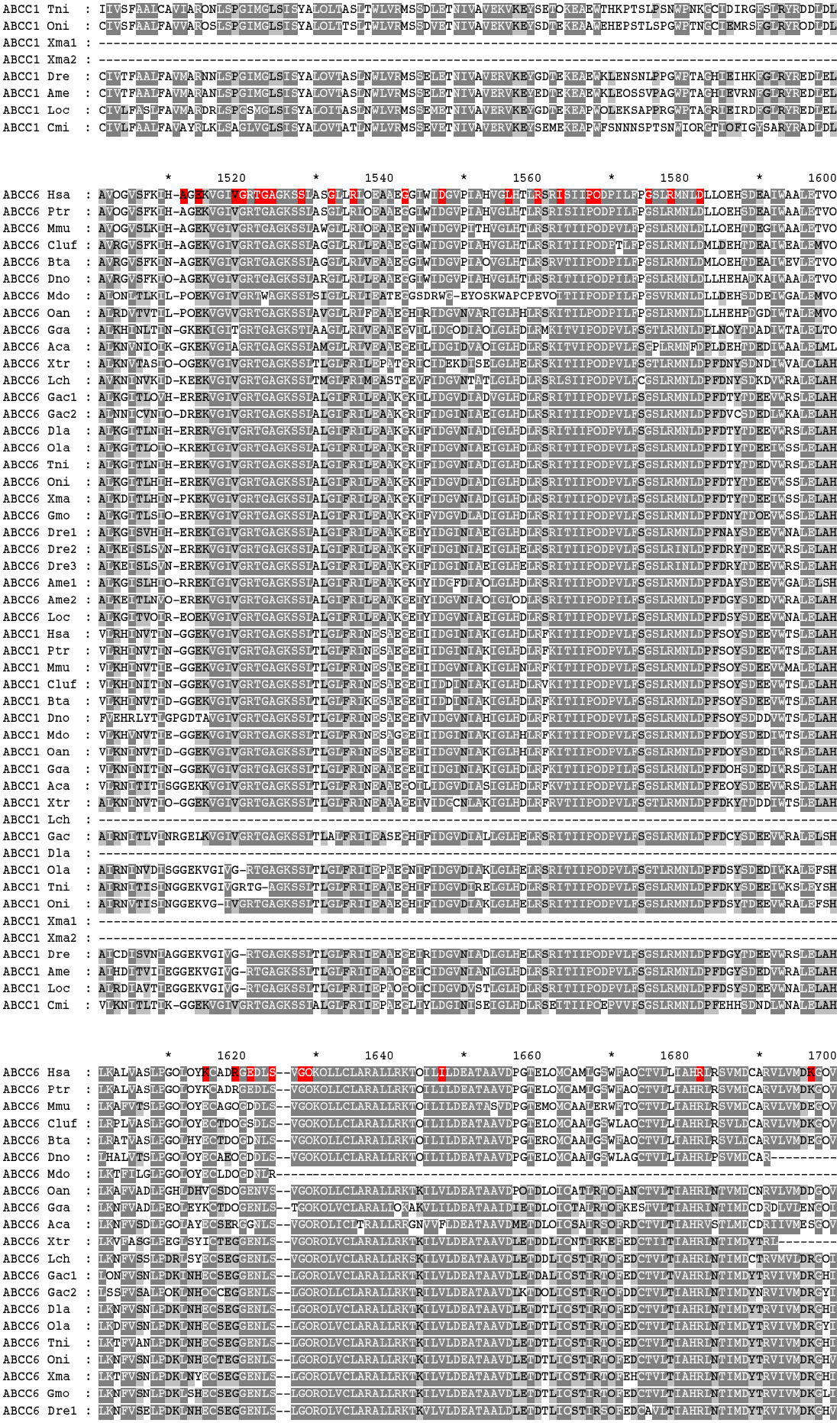


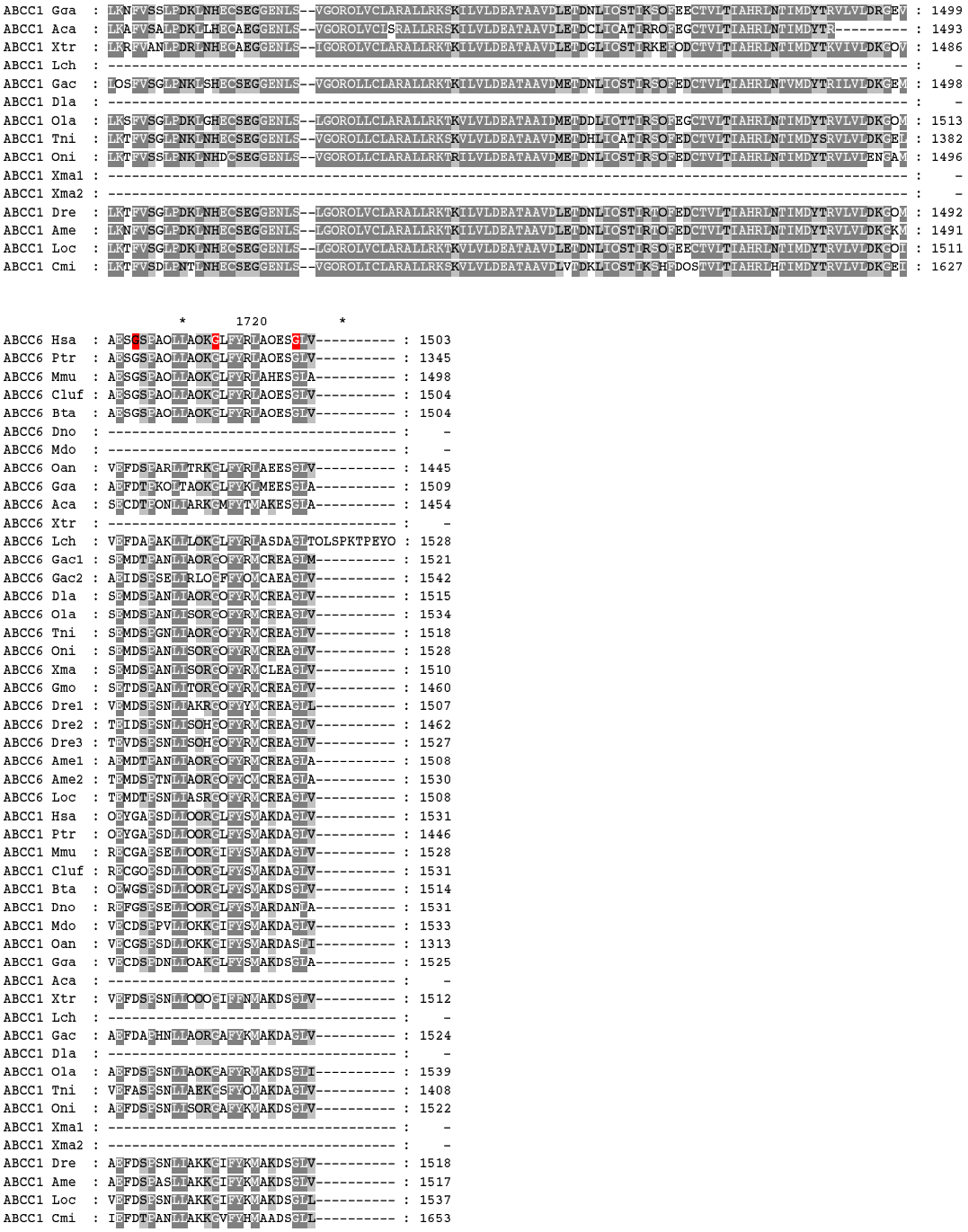




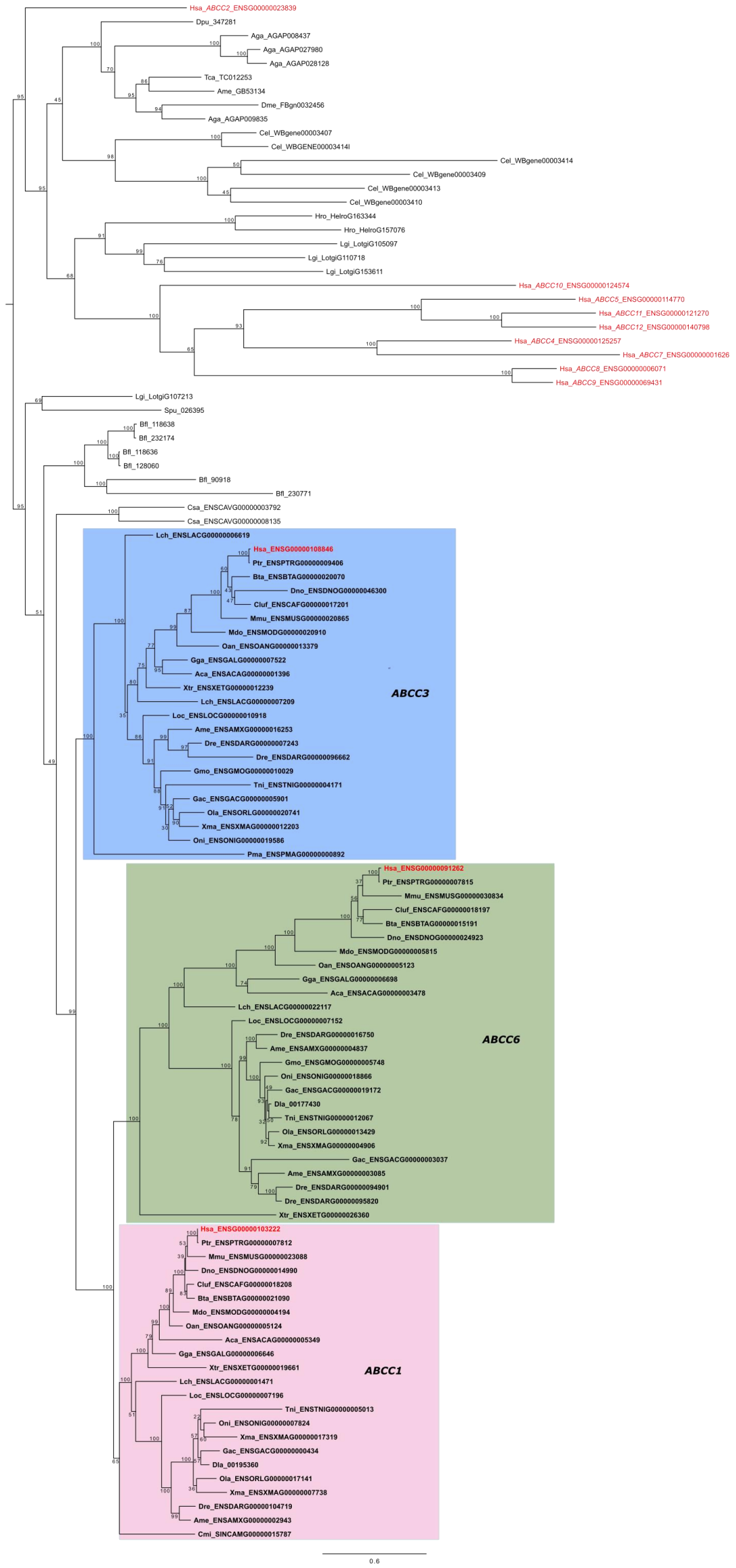








Supplementary figure 8-1. Sequence alignments of the MRP1 and MRP6 proteins in vertebrates. Conserved amino acids in the sequence alignment are shaded in dark grey. The selected 139 amino acids mutated in PXE disease analyzed in this study are coloured in red. Accession numbers are given in the supplementary table 8-1. Due to its size the alignment of all species (including invertebrates and the other ABCC family members) is not demonstrated but is available by request.



Supplementary figure 8-2. Phylogenetic tree of the vertebrate and invertebrate ABCC6, 1 and 3 with the other human ABCC family members. The phylogenetic tree was built using the ML method with PhyML 3.0 software with 100 bootstrap replicates using JTT+I+G+F substitution model given by Protest and was rooted using the invertebrate ABCC-like branch. To facilitate interpretation, the three major vertebrate ABCC clades are boxed with different colours.

Supplementary table 8-3. List of genes and their accession numbers used for linkage analysis. Data was retrieved from Ensembl Genomes genome database except the Elephant shark (*Callorhinchus milii*) orthologues that were obtained from (http://ensembl.fugu-sg.org/index.html, accessed August 2015).

Specie/Gene	***NDE1***	***MYH11***	***FOPNL***	***ABCC1***	***NOMO3***
Human	ENSG00000072864	ENSG00000133392	ENSG00000133393	ENSG00000103222	ENSG00000103226
Opossum	ENSMODG00000004385	ENSMODG00000004360	ENSMODG00000004204	ENSMODG00000004194	ENSMODG00000005682
Platypus	ENSOANG00000005121	ENSOANG00000005134	ENSOANG00000005132	ENSOANG00000005124	ENSOANG00000005122
Chicken	ENSGALG00000006500	ENSGALG00000006520	ENSGALG00000006532	ENSGALG00000006646	ENSGALG00000006730
Xenopus	ENSXETG00000005528	ENSXETG00000019650	ni	ENSXETG00000019661	ENSXETG00000019683
Coelacanth	ENSLACG00000006656	ENSLACG00000007990	ni	ENSLACG00000022117	ENSLACG00000012305
Zebrafish	ENSDARG00000037900	ENSDARG00000009782	ENSDARG00000071198	ENSDARG00000059874	ENSDARG00000078592
Stickleback	ENSGACG00000019165	ENSGACG00000019168	ENSGACG00000019170	ENSGACG00000000434	ENSGACG00000019191
See bass	DLAgn_00177400	DLAgn_00177410	DLAgn_00177420	DLAgn_00195360	DLAgn_00177480
Medaka	ENSORLG00000013552	ENSORLG00000013536	ENSORLG00000013435	ENSORLG00000017141	ENSORLG00000013299
Tetraodon	ENSTNIG00000011286	ENSTNIG00000012065	ENSTNIG00000012066	ENSTNIG00000005013	ENSTNIG00000012072
Spotted gar	ENSLOCG00000007273	ENSLOCG00000009053	ENSLOCG00000007248	ENSLOCG00000007196	ENSLOCG00000006964
Elephant Shark	SINCAMG00000015553	SINCAMG00000015577	SINCAMG00000015784	SINCAMG00000015787	SINCAMG00000015915
Specie/Gene	***ABCC6***	***XYLT1***	***RPS15A***	***ARL6IP1***	***COQ7***
Human	ENSG00000091262	ENSG00000103489	ENSG00000134419	ENSG00000170540	ENSG00000167186
Opossum	ENSMODG00000005815	ENSMODG00000005904	ENSMODG00000005920	ENSMODG00000005944	ENSMODG00000006166
Platypus	ENSOANG00000005123	ENSOANG00000009533	ENSOANG00000012228	ni	ENSOANG00000003221
Chicken	ENSGALG00000006698	ENSGALG00000006757	ENSGALG00000006771	ENSGALG00000006780	ENSGALG00000006861
Xenopus	ENSXETG00000026360	ENSXETG00000019693	ENSXETG00000002942	ENSXETG00000002943	ENSXETG00000002946
Coelacanth	ENSLACG00000001471	ENSLACG00000008838	ENSLACG00000011476	ENSLACG00000010670	ENSLACG00000005333
Zebrafish	ENSDARG00000016750	ENSDARG00000061248	ENSDARG00000010160	ENSDARG00000054578	ENSDARG00000062594
	ENSDARG00000094901				
	ENSDARG00000009582				
Stickleback	ENSGACG00000003037	ENSGACG00000019187	ENSGACG00000019181	ENSGACG00000014908	ENSGACG00000019175
	ENSGACG00000019172				
See bass	DLAgn_00177430	DLAgn_00177470	DLAgn_00177460	DLAgn_00194490	DLAgn_00177440
Medaka	ENSORLG00000013429	ENSORLG00000013339	ENSORLG00000013347	ENSORLG00000001074	ENSORLG00000013378
Tetraodon	ENSTNIG00000012067	ENSTNIG00000012071	ENSTNIG00000012070	ENSTNIG00000011302	ENSTNIG00000012068
Spotted gar	ENSLOCG00000007152	ENSLOCG00000006998	ENSLOCG00000007017	ENSLOCG00000007049	ENSLOCG00000007132
Elephant Shark	ni	SINCAMG00000015951	SINCAMG00000015952	SINCAMG00000015955	SINCAMG00000016019

Abbreviations: ni- not identified, *NDE1*- Neurodevelopment protein 1, *MYH11*- Myosin heavy chain 11, *FOPNL*- FOP related protein, *ABCC1,6*- ATP binding cassette subfamily C member 1,6, *NOMO3*- Nodal modulator 3, *XYLT1*- Xylosyltransferase 1, *RSPS15A*- Ribosomal protein S15A, *ARL6IP1*- ADP ribosylation factor like protein 6 interacting protein 1, *COQ7*- Coenzyme Q7.

Supplementary table 8-4. Percentage of amino acid sequence identity/similarity of the *ABCC6* and *ABCC1* sequences in comparison with human sequence by GeneDoc program.

Specie name	Common name	Abbreviation	% Identity/Similarity with Hsa	
			ABCC6	***ABCC1***
Homo sapiens	Human	Hsa	X	X
Pan troglodytes	Chimpanzee	Ptr	88/89	92/93
Mus musculus	Mouse	Mmu	78/86	87/95
Canis lupus familiaris	Dog	Cluf	80/89	91/96
Bos taurus	Cow	Bta	83/90	90/95
Dasypus novemcinctus	Armadillo	Dno	73/81	86/93
Monodelphis domestica	Opossum	Mdo	57/70	83/91
Ornithorhynchus	Platypus	Oan	59/74	71/78
Gallus gallus	Chicken	Gga	52/69	77/89
Anolis carolinensis	Anole Lizard	Aca	50/68	68/80
Xenopus tropicalis	Xenopus	Xtr	40/60	69/82
Latimeria chalumnae	Coelacanth	Lch	48/67	49/59
Gasterosteus aculeatus	Stickleback	Gac	44/64	66/80
			43/60	
Dicentrarchus labrax	See bass	Dla	42/60	36/44
Oryzias latipes	Medaka	Ola	45/63	64/80
Tetraodon nigroviridis	Tetraodon	Tni	45/64	52/66
Oreochromis niloticus	Tilapia	Oni	45/63	65/81
Xiphophorus maculatus	Platyfish	Xma	45/64	12/16
				24/29
Gadus morhua	Cod	Gmo	43/62	XX
Danio rerio	Zebrafish	Dre	45/64	68/81
			44/62	
			44/62	
Astyanax mexicanus	Blind cave fish	Ame	46/64	68/81
			44/62	
Lepisosteus oculatus	Spotted gar	Loc	47/65	68/81
Callorhinchus milii	Elephant shark	Cmi	X	56/71
Petromyzon marinus	Lamprey	Pma	X	X

Supplementary table 8-5. Size of the exons and introns deduced from Ensembl for the Human and spotted gar *ABCC6* orthologues genes. The sizes of human pseudogenes are also indicated. Bold represents the exons with the same size than human *ABCC6* gene (accessed August 2015).

Exons	Human (Chr16)			Spotted gar (LG13)
	ABCC6	***ABCC6P1***	***ABCC6P2***	***abcc6***
E1	66	124	36	42
I1	1567	1565	1565	4807
E2	183	184	183	177
I2	1701	1259	1259	354
E3	126	116	116	126
I3	139	325	325	2569
E4	129	126	126	138
I4	5104	139	139	3025
E5	126	129	129	126
I5	2077	5055	ni	1531
E6	62	126	ni	62
I6	3325	2070	ni	802
E7	132	62	ni	132
I7	5114	3338	ni	897
E8	204	132	ni	201
I8	1231	5166	ni	1105
E9	178	204	ni	178
I9	3818	1231	ni	126
E10	162	178	ni	162
I10	5098	4050	ni	335
E11	93	1562	ni	93
I11	2462	ni	ni	989
E12	204	ni	ni	204
I12	1189	ni	ni	256
E13	114	ni	ni	147
I13	1619	ni	ni	304
E14	88	ni	ni	88
I14	2089	ni	ni	865
E15	127	ni	ni	70
I15	215	ni	ni	114
E16	177	ni	ni	127
I16	3446	ni	ni	308
E17	177	ni	ni	177
I17	3446	ni	ni	609
E18	168	ni	ni	168
I18	1171	ni	ni	423
E19	175	ni	ni	184
I19	1465	ni	ni	268
E20	76	ni	ni	82

Exons	Human (Chr16)			Spotted gar (LG13)
	ABCC6	***ABCC6P1***	***ABCC6P2***	***abcc6***
I20	2506	ni	ni	108
E21	121	ni	ni	118
I21	3430	ni	ni	237
E22	208	ni	ni	208
I22	3712	ni	ni	572
E23	311	ni	ni	311
I23	2430	ni	ni	438
E24	200	ni	ni	200
I24	1428	ni	ni	223
E25	127	ni	ni	127
I25	1854	ni	ni	282
E26	102	ni	ni	102
I26	1672	ni	ni	340
E27	147	ni	ni	147
I27	2631	ni	ni	349
E28	159	ni	ni	159
I28	78	ni	ni	403
E29	167	ni	ni	167
I29	3855	ni	ni	174
E30	195	ni	ni	195
I30	336	ni	ni	691
E31	109	ni	ni	109

Abbreviations: Chr- chromosome.

Supplementary table 8-6. CT values for the control gene *rps18* and *abcc6* and *abcc1* genes in intact and regenerating skin at 0-96 hours.

TIME	Intact skin			Regenerating skin		
	Control	***abcc6***	***abcc1***	**Control**	***abcc6***	***abcc1***
0h	25,96	28,65	31,71	--	--	--
6h	25,47	28,27	33,06	25,10	27,35	33,58
24h	25,54	27,72	31,22	24,07	26,49	30,86
48h	26,09	27,07	31,40	24,85	26,36	30,35
72h	24,80	26,41	29,83	24,26	25,84	29,40
96h	25,72	26,45	30,51	25,25	25,85	30,95

CHAPTER IX: GENERAL DISCUSSION AND CONCLUSIONS

9. GENERAL DISCUSSION AND CONCLUSIONS

This study was conducted in the Azores, a Portuguese archipelago located in the middle of the Atlantic Ocean, in a small island with only 56.467 inhabitants (Census, 2011). The high rate of consanguineous marriage and the high probability of common ancestry increases the possibility of finding diseases caused by a single gene. The coexistence of DISH with Chondrocalcinosis (CC) is very common on this island and seems to be an endemic manifestation. Previous studies, undertaken by our group, led to the identification and characterization of twelve families with early onset CC and/or DISH leading the group to suggest that both diseases, designated as DISH/CC phenotype, could share the same pathogenic mechanism [23]. These families may represent a familial type of pyrophosphate arthropathy with a phenotype that includes peripheral and axial enthesopathic calcifications. A similar phenotype has been described in several other studies in different populations [25, 26]. The meticulous analysis of twelve pedigrees pointed toward a Mendelian disease with autosomal-dominant mode of inheritance [23]. Eight of the probands of these families were born in the same or nearby villages and this phenotype has higher in specific zones of the island, raising the possibility of an unknown environmental factor or a genetic founder effect. In fact, founder effects have been used to explain the presence of high-frequency Mendelian diseases in many isolated populations [456, 457]. Since isolated populations have proven particularly valuable for the purposes of mapping genes involved in rare Mendelian monogenic disorders, studying DISH/CC in this island population will increase the likelihood of identifying the causative mutation. For a number of years the group that hosted the present study has been looking for a possible major gene in the aethiopathogenesis of the DISH/CC phenotype. The involvement of the *ANKH* gene, the only monogenic disease causing gene yet known for chondrocalcinosis, has already been discarded by a previous study [110]. A whole genome linkage analysis followed by an “Identity-by-state/descent” was performed and two zones, in chromosomes 12 and 20, seemed relevant for further investigation. In line with these results, two genes were considered to be good candidate genes for DISH/CC; *RSPO4* on chromosome 20, and *LEMD3* on chromosome 12. Several gene variants were identified and nucleotide modifications located in the regulatory region of the *RSPO4* gene were more frequent in controls than in the DISH/CC group (p-values; 0.03 and 0.05). *RSPO4* mutations, especially those located in the highly conserved exons 2 and 3 of the gene encoding the furin/like cysteine/rich domain of R/spondin 4, causes autosomal-recessive anonychia [351]. The specific variant Gly67Arg causes anonychia by disrupting the Wnt/β-catenin signaling

pathway [458]. The effects on the protein of the two regulatory variants found in our study have not been defined. However, the variants are located in the regulatory region of the gene and so may alter the expression of the *RSPO4* gene, although the modification in gene expression and why these variants are protective to DISH/CC is unknown. According to our results, neither *RSPO4* nor *LEMD3* are the main genetic causes for the development of the DISH/CC phenotype. These genes, in particular *RSPO4* may, however, have a modest role which should be further investigated.

To further identify candidate genes or variants associated with DISH/CC phenotype we performed Whole Exome Sequencing on four unrelated DISH/CC patients, followed by association studies of selected variants. Our focus on the "exome" was due to the common belief that the coding regions contain the most functionally relevant variants and because many coding mutations have been found to cause phenotypic effects [459]. For instance, missense mutations in *ANKH* gene causing gain or loss of function of ANK protein leads to the development of chondrocalcinosis (CCAL2; MIM 118600) [343-345] or Craniometaphyseal dysplasia (CMDD; MIM #123000) [109, 346], respectively. Other example is the coding mutations in the *ACVR1* which cause a disruption of the glycine/serine-rich domain of the activin A receptor 1 leading to the progressive ossification disease (FOP; MIM #135100) [460, 461]. Mutations in *ABCC6* gene normally occur in cytoplasmatic regions of the protein [406], and cause a loss of function of the protein leading to the Pseudoxanthoma elasticum disease (PXE; MIM #264800) [126]. In fact the mutations that cause Mendelian diseases occur primarily in coding regions, and mutations that causes amino acids substitutions are the most frequent type of disease mutations (~60%) [462]. The candidate genes selected for the analysis in this study were those directly or indirectly related to mineralization or ossification, in which coding or splice-site mutations were found.

BMP4 is a member of the BMP family and belongs to the transforming growth factor- β (TGF-β) superfamily, which is involved in osteoblast differentiation and bone formation [463]. One common missense variant in this gene, rs17563 T>C (p.V152A), was found in three out of four of the investigated patients (WES). After typing this variant in a group of DISH/CC patients and controls we observed that the C allele is statistically more frequent in the DISH/CC group than in controls. This variant is very frequent in all populations (MAF=0.37) which does not invalidate a role on the susceptibility to DISH/CC, since common variants associated with disease risk are not uncommon. In OPLL disease, for

instance, the majority of susceptibility variants associated are common variants [61] [33, 146] [61]. The rs17563 is one of the most functional SNP of *BMP4* gene [464]. This SNP causes a change in the mRNA structure and the levels of BMP4 mRNA are significantly higher in T-allele carriers when compared with C-allele carriers [381]. The T-allele has been associated with OPLL disease [160, 161], and mRNA and expression of BMP4 protein were significantly increased in OPLL cells derived from ossified spinal ligament when compared to non OPLL cells [382]. The rs17563 variant has been largely studied as associated with non-syndromic cleft lip with or without palate (NSCL/P), however the results were inconsistent. For some the C allele is a risk factor for the disease [465, 466] and for others the C allele is protective [467]. To clarify this association a meta-analysis was performed and the authors conclude that the rs17563 variant could play a different role during the development of NSCL/P based on ethnicity diversity [464]. NSCL/P is a complex genetic disorder with a variable phenotype, largely attributed to the interactions of the environment and multiple genes, each potentially having certain effects [468]. In our case it is very difficult to state that this single variant is the responsible for the DISH/CC phenotype, but rather this variant could contribute to the DISH/CC phenotype by interacting with other moderate effect genes and/or with unknown enviromental factors. At this moment we do not know how the C variant contributes to the DISH/CC phenotype and thus there is a need for further research in this area.

The main candidate gene investigated in this thesis was the *ABCC6* gene, a member of the ATP-Binding Cassette family of transporters that has been extensively related to dermal ectopic calcification and has recently become a new member of the calcification regulators in mammals [122]. Loss-of-function mutations within the *ABCC6* gene cause PXE and in some cases GACI, both heritable disorders related to soft tissue calcification. PXE and GACI are rare disorders and none of the patients investigated in this study were diagnosed with these conditions. The exact function of the protein encoded by this gene and the substrate(s) that it transport remain unknown [469], but evidences suggests that it may be involved in the metabolism of pyrophosphate. In PXE disease MRP6 deficiency leads to strong reduced plasma PPi levels that lead to a reduced PPi/Pi ratio and this favours pathological mineralization [397]. The same occurs in *abcc6*$^{-/-}$ mice, in which PPi levels were lower than in wild type mice [397]. It is now widely accepted that Pi and PPi levels are determinants for the development of rheumatic calcifying disorders. One missense rare variant in the *ABCC6* gene, rs41278174 C>T (p. R1064W), was present in one of the DISH/CC patients. A large

number of other variants were found in this gene, but no differences were identified between DISH/CC and controls. The rs41278174 variant is highly conserved between mammals, and according to the algorithms used (SIFT and PolyPhen) is deleterious and damaging to the protein. We observed that this variant had a higher frequency in the DNA samples of our island population and it was more frequent in male controls than in male patients with disorders that are characterized by ectopic calcification, such as DISH/CC and Ankylosing Spondylitis. This variant is located in the transmembrane domain of the MRP6 protein, which plays a role in the substrate specificity of ABC transporters [412]. Mutations in the transmembrane domain can also affect the integration of the protein into the cell membrane leading to a loss of function [308]. We hypothesize that the variant - rs41278174 - in males, may change the specificity of the MRP6 transporter, conferring a protective effect via an unknown mechanism.

In humans, the *ABCC6* gene has highest expression in the liver and kidneys and localizes to the plasma membrane [470]. However, several other studies show that *ABCC6* is expressed in other tissues as well, albeit in much lower abundance, including the skin and the blood vessel walls, which are sites of pathology in PXE [125, 471, 472]. In fish, the *abcc6a* gene functions locally at the site of mineralization and in zebrafish the gene is also expressed in intervertebral disc regions, structures that are affected by hypermineralization caused by a specific mutation in *abcc6a* gene [113]. In this study, we found expression of *ABCC6* in human cartilage tissues and the gene was also expressed in fish skin (normal and regenerating). In fish skin, no difference was found between normal skin and regenerating skin, and the function of this gene in fish skin remains an open question. The retention of the gene in the genome of the bony fishes but its loss from the genome of the cartilaginous fish, from which they derived, suggests that the gene has an important function. Furthermore, comparative genomics analysis indicated that the *abbc6* gene emerged at the same time as bone vertebrates indicating it is most likely linked to specific innovations in the vertebrates.

In human cartilage tissues we found very different levels of the *ABCC6* transcript between patients. When grouped by disease we found that, in general, the level of expression was increased in coxoarthrosis, and was more evident in DISH and CC patients when compared with the only available control. In vitro, the overexpression of *ABCC6* induces nucleotide release [129]. Although MRP6 does not directly transport ATP, it is thought that ATP is secreted via an MRP6 dependent mechanism. In the liver, ATP is converted into AMP and

PPi by ENPP1 and PPi is the main inhibitor of mineralization, in plasma [397]. The Pi:PPi ratio is a determinant factor that can lead to pathological mineralization or its inhibition [473]. In general, conditions which favour PPi increase promote CPP crystal formation, while decreases in PPi promotes hydroxyapatite crystal formation [474]. In line with this hypothesis we postulate that, in our patients, the PPi levels are higher, favouring CPP formation instead of hydroxyapatite crystals. In CC the deposited salts are normally composed of CPP, though other calcium salts can also be found including hydroxyapatite [256, 475]. In some cases, CPP can also occur in the spinal ligaments [257, 258], but it is usually difficult to distinguish from ossification [46]. In fact, in our patients the involvement of CPP deposition was already known, since CPP crystals were identified from knee effusions in 13 patients from the 12 DISH/CC families previously characterized [24].

Putting it all together, our results raise a question in relation to the monogenic or polygenic nature of the DISH/CC disease coupled with the possible involvement of environmental factors. The association of the rs17563 variant in the *BMP4* gene, found in our study, has probably a minor effect on disease development. As suggested for OPLL [152], it seems that the association of different genes and variants are important and that it is unlikely that there is only one major gene responsible for the disease. Rather, it seems most likely that a number of variants in a variety of different genes may be associated with DISH, and therefore predispose for the development of the disease in patients. The former facts explain the failure in previous studies to identify causative genes, as the studies assumed a single gene inheritance pattern.

The studies carried out and reported in this thesis have several limitations **1)** DISH is currently lacking a validated set of diagnostic and classification criteria in order to better describe and establish homogeneous cohorts of patients. This is particularly important because DISH is characterized not only by the ossification of the anterior spinal ligaments but also by generalized symmetrical enthesopathic calcifications. Much has been debated about the importance of diagnostic and classification criteria for DISH, but no agreement has been reached so far [40]. In our opinion, a well validated set of classification criteria for DISH, is of extreme importance for genetic studies in order to have a homogeneous phenotype group for investigations; **2)** the small sample size, especially the control group with only 36 individuals gives very low statistical power of the study. The low sample number for the control group is linked to the need in the asymptomatic population to carry out radiographic examinations to identify those affected by the pathology; **3)** All the filtering strategies

employed involved previous knowledge on the genes. The biggest limitation in this strategy is that genes that were not labeled yet or have unknown functions were not investigated in this study and may be relevant; **4)** of course it cannot be assumed that any gene variant will necessarily have a negative impact on protein expression and function, but by focusing on the coding region we aimed to eliminate the synonymous and intronic variants. Normally the variants in the human genome with deleterious effects on the protein are the basis for the development of diseases, however variations that modify protein structure do not necessarily lead to a detectable human-disease phenotype, and a mutation that predispose an individual to a disease is not necessarily a deleterious variation. The synonymous variants are now widely acknowledged to cause changes in protein expression, conformation and function [476]. Sometimes the altered nucleotide is part of a splicing enhancer or suppressor and the change affects splicing or in other cases they do not affect splicing but the alternative codon could require an alternative transfer RNA that is in short supply, and this therefore changes the kinetics of translation [477]. For instance synonymous variants in the *CFTR* gene causes aberrant splicing in patients with Cystic Fibrosis [478] [479]. Thus, synonymous mutations within protein-coding regions may be associated with noncoding functions, acting pre-transcriptionally at the DNA level or post-transcriptionally at the RNA level [480]. An example of this is found with the dopamine receptor gene (*DRD2)* in which a synonymous variant is associated with schizophrenia and alcoholism by modulating receptor production through differences in messenger RNA (mRNA) folding and stability [481]. Also the intronic variants could be of interest. Transcript variants within an intron may regulate genes; through modifying alternative splicing of the mRNA or by changing the binding site of enhancers that act on the gene they are found in or could possibly enhance the expression of many genes. Disease associated intronic variants in the *ErbB4* gene are related to altered ErbB4 splice-variant expression in the brains in schizophrenia [482]; **5)** the study population comprised only Azorean individuals and we cannot generalize our conclusion to other ethnic populations. Thus, the present findings need to be replicated and validated in a greater number of samples including other ethnic groups. The public health risk posed by the risk alleles is likely to show wide variation across populations simply as a function of its frequency, and this risk difference may be amplified by gene-gene and gene-environment interactions; **6)** our expression studies were preliminary and with a reduced sample size.

In the future further studies will be required to overcome the limitation of the present study this could include; **1)** arranging groups according to the calcified tissue regions, since there is no exact classification criteria for DISH currently available, obviously this will greatly

decrease the sample size, and thus more patients will be necessary; **2)** look more closely at the unknown genes and to the synonymous and intronic variants obtained in the WES results in order to verify if there is a possible association with DISH/CC (extra filtering of the results); **3)** perform additional studies with an enlarged number of samples and expression studies to clarify the association of the *BMP4* gene variant (rs17563) with our phenotype; **4)** confirm if *ABCC6* overexpression is related to the formation of CPP deposition in DISH and CC patients. Clearly in the future it will be important to extend the biobank and collect the necessary cartilage samples to ensure a bigger sample size that will generate more robust data.

In conclusion, the findings of our study lead us to suggest that DISH/CC is polygenic, being influenced by the interaction of multiple small effect gene variants and possibly by unknown environmental factors. Exome sequencing combined with Sanger sequencing is confirmed by this study to be an efficient strategy to search for disease causing genes of both monogenic and polygenic diseases. Therefore, it seems most likely that DISH/CC phenotype has many potential different mutations in various genes in different chromosomes involved in its inheritance, pathology, and expression.

CHAPTER X: BIBLIOGRAPHY

10. BIBLIOGRAPHY

1. Mader, R., *Clinical manifestations of diffuse idiopathic skeletal hyperostosis of the cervical spine.* Sem Arthritis Rheum 2002. **32**(2): p. 130-5.
2. Mader, R., J.J. Verlaan, and D. Buskila, *Diffuse idiopathic skeletal hyperostosis: clinical features and pathogenic mechanisms.* Nat Rev Rheumatol, 2013. **9**(12): p. 741-50.
3. Mader, R., et al., *Extraspinal manifestations of diffuse idiopathic skeletal hyperostosis.* Rheumatology (Oxford), 2009. **48**(12): p. 1478-81.
4. Verlaan, J.J., et al., *Diffuse idiopathic skeletal hyperostosis of the cervical spine: an underestimated cause of dysphagia and airway obstruction.* Spine J, 2011. **11**(11): p. 1058-67.
5. Utsinger, P.D., *Diffuse Idiopathic Skeletal Hyperostosis.* Clin Rheum Dis, 1985. **11**(2): p. 325-51.
6. Resnick, D., et al., *Association of diffuse idiopathic skeletal hyperostosis (DISH) and calcification and ossification of the posterior longitudinal ligament.* Am J Roentgenol 1978. **131**(6): p. 1049-53.
7. Williamson, P.K. and A.J. Reginato, *Diffuse idiopathic skeletal hyperostosis of the cervical spine in a patient with ankylosing spondylitis.* Arthritis Rheum, 1984 **27**(5): p. 570-3.
8. Olivieri, I., et al., *Coexistence of ankylosing spondylitis and diffuse idiopathic skeletal hyperostosis: another report.* J Rheumatol., 1987. **14**(5): p. 1058-60.
9. Olivieri, I., et al., *Concomitant ankylosing spondylitis and DISH.* J Rheumatol, 1989 **16**(8): p. 1170-2.
10. Rillo, O.L., et al., *Coexistence of diffuse idiopathic skeletal hyperostosis and ankylosing spondylitis.* Clin Rheumatol., 1989 **8**(4): p. 499-503.
11. Troise Rioda, W. and G.F. Ferraccioli, *DISH and ankylosing spondylitis. Case report and review of the literature.* Clin Exp Rheumatol., 1990 **8**(6): p. 591-3.
12. Passiu, G., G.D. Sebastiani, and M.A. Tuveri, *[Diffuse idiopathic skeletal hyperostosis of the cervical spine in a patient with ankylosing spondylitis. Description of a personal case]*. Ann Ital Med Int., 1991 **6**(1 Pt 1): p. 61-4.
13. Olivieri, I., *Coexisting diffuse idiopathic skeletal hyperostosis and ankylosing spondylitis.* Clin Rheumatol., 1991 **10**(1): p. 95.
14. Tishler, M. and M. Yaron, *Two cases of diffuse idiopathic skeletal hyperostosis and ankylosing spondylitis.* Br J Rheumatol., 1992 **31**(8): p. 569-71.
15. Maertens, M., et al., *Simultaneous occurrence of diffuse idiopathic skeletal hyperostosis and ankylosing spondylitis in the same patient.* J Rheumatol., 1992 **19**(12): p. 1978-83.
16. Jattiot, F., P. Goupille, and J.P. Valat, *Coexistence of diffuse idiopathic skeletal hyperostosis and ankylosing spondylitis in two patients.* Clin Exp Rheumatol., 1995 **13**(3): p. 405-6.
17. Moreno, A.C., et al., *Simultaneous occurrence of diffuse idiopathic skeletal hyperostosis and ankylosing spondylitis.* Rev Rhum Engl Ed., 1996 **63**(4): p. 292-5.
18. Kozanoglu, E., et al., *Coexistence of diffuse idiopathic skeletal hyperostosis and ankylosing spondylitis: a case report.* Clin Rheumatol., 2002 **21**(3): p. 258-60.
19. Wooten, M., *The coexistence of ankylosing spondylitis and diffuse idiopathic skeletal hyperostosis.* Clin Rheumatol., 2009. **28**(5): p. 619.
20. Jordana, X., et al., *The coexistence of ankylosing spondylitis and diffuse idiopathic skeletal hyperostosis--a postmortem diagnosis.* Clin Rheumatol., 2009 **28**(3): p. 353-6.

21. Macia-Villa, C.C., W.A. Sifuentes-Giraldo, and J. Medina-Luezas, *Simultaneous occurrence of ankylosing spondylitis and diffuse idiopathic skeletal hyperostosis (Forestier-Rotes-Querol disease).* Reumatol Clin, 2016.
22. Resnick, D., et al., *Diffuse Idiopathic Skeletal Hiperostosis (DISH) [Ankylosing Hyperostosis of Forrestier and Rotes- Querol]*. Sem Arthritis Rheum, 1978. **7**(3): p. 153-87.
23. Bruges-Armas, J.B., et al., *Ectopic Calcification in the Azores - Clinical and radiological manifestations of DISH and Chondrocalcinosis Families* Arthritis Rheum, 2006. **54**(4): p. 1340-1349.
24. Bruges-Armas, J., et al., *Ectopic calcification among families in the Azores: clinical and radiologic manifestations in families with diffuse idiopathic skeletal hyperostosis and chondrocalcinosis.* Arthritis Rheum, 2006. **54**(4): p. 1340-9.
25. van der Korst, J.K., J. Geerards, and F.C. Driessens, *A hereditary type of idiopathic articular chondrocalcinosis. Survey of a pedigree.* Am J Med, 1974. **56**(3): p. 307-14.
26. Okazaki, T., et al., *Pseudogout: clinical observations and chemical analyses of deposits.* Arthritis Rheum, 1976. **19 Suppl 3**: p. 293-305.
27. Parreira, B., *Biobanco «AZORBIO»: organização de recursos biológicos para a investigação*. 2012, Universidade dos Açores: Ponta Delgada. p. Vol. 1: 59 f.; Vol. 2: 100 f.
28. Altman, R.D., et al., *Articular chondrocalcinosis. Microanalysis of pyrophosphate (PPi) in synovial fluid and plasma.* Arthritis Rheum, 1973. **16**(2): p. 171-8.
29. Couto, A.R., *Genetics and Epidemiology of ectopic calcification*, in *Biomedical Sciences*. 2009, University of Porto: Porto. p. 234.
30. Santos, C., et al., *Genetic structure of the Azores Islands: a study using 15 autosomal short tandem repeat loci.* Coll Antropol, 2009. **33**(4): p. 991-9.
31. Ikegawa, S., *Genomic study of ossification of the posterior longitudinal ligament of the spine.* Proc Jpn Acad Ser B Phys Biol Sci, 2014. **90**(10): p. 405-12.
32. Guo, Q., et al., *Simultaneous ossification of the posterior longitudinal ligament and ossification of the ligamentum flavum causing upper thoracic myelopathy in DISH: case report and literature review*. Eur Spine J, 2011. **20 Suppl 2**: p. S195-201.
33. Kong, Q., et al., *COL6A1 polymorphisms associated with ossification of the ligamentum flavum and ossification of the posterior longitudinal ligament.* Spine (Phila Pa 1976), 2007. **32**(25): p. 2834-8.
34. Tsukahara, S., et al., *COL6A1, the candidate gene for ossification of the posterior longitudinal ligament, is associated with diffuse idiopathic skeletal hyperostosis in Japanese*. Spine, 2005 **30**(20): p. 2321-4.
35. Liu, Y., et al., *RUNX2 polymorphisms associated with OPLL and OLF in the Han population.* Clin Orthop Relat Res, 2010. **468**(12): p. 3333-41.
36. Knaggs, R.L., *Spondylitis deformans.* Br J Surg, 1925. **12**: p. 524-546.
37. Forestier, J. and J. Rotes-Querol, *Senile ankylosing hyperostosis of the spine.* Ann Rheum Dis, 1950. **9**: p. 321-30. .
38. Beyeler, C., et al., *Diffuse idiopathic skeletal hyperostosis (DISH) of the elbow: a controlled radiological study.* BMC Musculoskelet Disord, 2015. **16**: p. 119.
39. Resnick, D. and G. Niwayama, *Radiographic and pathologic features of spinal involvement in diffuse idiopathic skeletal hyperostosis (DISH).* Radiology, 1976. **119**: p. 559.
40. Mader, R., et al., *Developing new classification criteria for diffuse idiopathic skeletal hyperostosis: back to square one.* Rheumatology (Oxford), 2013. **52**(2): p. 326-30.

41. Yoshimura, N., et al., *Prevalence and progression of radiographic ossification of the posterior longitudinal ligament and associated factors in the Japanese population: a 3-year follow-up of the ROAD study.* Osteoporos Int, 2014. **25**(3): p. 1089-98.
42. Abiola, R., P. Rubery, and A. Mesfin, *Ossification of the Posterior Longitudinal Ligament: Etiology, Diagnosis, and Outcomes of Nonoperative and Operative Management.* Global Spine J, 2016. **6**(2): p. 195-204.
43. Ohtsuka, K., et al., *An epidemiological survey on ossification of ligaments in the cervical and thoracic spine in individuals over 50 years of age.* Nihon Seikeigeka Gakkai Zasshi, 1986. **60**(11): p. 1087-98.
44. Tanaka, M., A. Kanazawa, and K. Yonenobu, *Diagnosis of OPLL and OYL: overview*, Springer, Editor. 2006: Tokyo, Japan. p. 111-114.
45. Epstein, N.E., *What you need to know about ossification of the posterior longitudinal ligament to optimize cervical spine surgery: A review.* Surg Neurol Int, 2014. **5**(Suppl 3): p. S93-S118.
46. Ehara, S., et al., *Paravertebral ligamentous ossification: DISH, OPLL and OLF*. Eur J Radiol, 1998. **27**(3): p. 196-205.
47. Kawaguchi, Y., et al., *New classification system for ossification of the posterior longitudinal ligament using CT images.* J Orthop Sci, 2014. **19**(4): p. 530-6.
48. Miyasaka, K., et al., *Ossification of spinal ligaments causing thoracic radiculomyelopathy.* Radiology, 1982. **143**(2): p. 463-8.
49. Yamagami, T., N. Kawano, and H. Nakano, *Calcification of the cervical ligamentum flavum--case report.* Neurol Med Chir (Tokyo), 2000. **40**(4): p. 234-8.
50. Brown, T.R., S.F. Quinn, and A.N. D'Agostino, *Deposition of calcium pyrophosphate dihydrate crystals in the ligamentum flavum: evaluation with MR imaging and CT.* Radiology, 1991. **178**(3): p. 871-3.
51. Ellman, M.H., et al., *Calcium pyrophosphate deposition in ligamentum flavum.* Arthritis Rheum, 1978. **21**(5): p. 611-3.
52. Mwaka, E.S., et al., *Calcium pyrophosphate dehydrate crystal deposition in the ligamentum flavum of the cervical spine: histopathological and immunohistochemical findings.* Clin Exp Rheumatol, 2009. **27**(3): p. 430-8.
53. Sucu, H.K. and B. H., *Ossification of the ligamentum flavum at the upper thoracic spine: Case report.* Turk Neurosurg, 2004(14): p. 49-51.
54. Fotakopoulos, G., et al., *Ossification of the ligamentum flavum in cervical and thoracic spine. Report of three cases.* Acta Neurol Belg, 2010. **110**(2): p. 186-9.
55. Cassim, B., G.M. Mody, and D.L. Rubin, *The prevalence of diffuse idiopathic skeletal hyperostosis in African blacks.* Br J Rheumatol, 1990. **29**(2): p. 131-2.
56. Weinfeld, R.M., et al., *The prevalence of diffuse idiopathic skeletal hyperostosis (DISH) in two large American Midwest metropolitan hospital populations.* Skeletal Radiol 1997. **26**(4): p. 222-5.
57. Holton, K.F., et al., *Diffuse idiopathic skeletal hyperostosis and its relation to back pain among older men: the MrOS Study.* Semin Arthritis Rheum, 2011. **41**(2): p. 131-8.
58. Hirasawa, A., et al., *The prevalence of diffuse idiopathic skeletal hyperostosis in Japan - the first report of measurement by CT and review of the literature.* J Orthop Sci, 2016. **21**(3): p. 287-90.
59. Kiss, C., et al., *Prevalence of diffuse idiopathic skeletal hyperostosis in Budapest, Hungary.* Rheumatology (Oxford), 2002. **41**(11): p. 1335-6.
60. Matsunaga, S. and T. Sakou, *Epidemiology of ossification of the posterior longitudinal ligament*. Ossification of the posterior longitudinal ligament ed. K. Yonenobu, T. Sakou, and K. Ono. 1997, Tokio: Springer. 11-7.

61. Maeda, S., et al., *Gender-specific haplotype association of collagen alpha2 (XI) gene in ossification of the posterior longitudinal ligament of the spine.* J Hum Genet, 2001. **46**(1): p. 1-4.
62. Fujimori, T., et al., *Prevalence, Concomitance, and Distribution of Ossification of the Spinal Ligaments: Results of Whole Spine CT Scans in 1500 Japanese Patients.* Spine (Phila Pa 1976), 2016.
63. Terayama, K., *Genetic studies on ossification of the posterior longitudinal ligament of the spine.* Spine (Phila Pa 1976), 1989. **14**(11): p. 1184-91.
64. Beardwell, A., *Familial Ankylosing Vertebral Hyperostosis With Tylosis.* Ann Rheum Dis, 1969. **28**: p. 518-23.
65. Abiteboul, M., B. Mazières, and H. Ménard, *À Propos De Deux Nouveaux Cas Familiaux D`Hyperostose Vertébrale Ankylosante* Rev Rhumatisme, 1985 **52**: p. 645-7.
66. Gorman, C., A.S.M. Jawad, and I. Chikanza, *A family with diffuse idiopathic skeletal hyperostosis.* Ann Rheum Dis, 2005. **64**: p. 1794-95.
67. Fornaciari, G., et al., *The 'gout' of the Medici, Grand Dukes of Florence: a palaeopathological study.* Rheumatology (Oxford), 2009. **48**(4): p. 375-7.
68. Hamanishi, C., et al., *Ossification of the posterior longitudinal ligament. Autosomal recessive trait.* Spine (Phila Pa 1976), 1995. **20**(2): p. 205-7.
69. Tanikawa, E., K. Furuya, and H. Nakajima, *Genetic study on ossification of posterior longitudinal ligament.* Bull Tokyo Med Dent Univ, 1986. **33**(3): p. 117-28.
70. Koga, H., et al., *Genetic mapping of ossification of the posterior longitudinal ligament of the spine.* Am J Hum Genet, 1998. **62**(6): p. 1460-7.
71. Tanabe, J., H. Sarin, and G. Kindt, *Familial ossification of the posterior longitudinal ligament (OPLL) in the thoracic spine: case report.* Surg Neurol, 2002. **58**(6): p. 403-5; discussion 405.
72. Kim, K.H., et al., *Association between BMP-2 and COL6A1 gene polymorphisms with susceptibility to ossification of the posterior longitudinal ligament of the cervical spine in Korean patients and family members.* Genet Mol Res, 2014. **13**(1): p. 2240-7.
73. Kranenburg, H.C., et al., *The dog as an animal model for DISH?* Eur Spine J, 2010. **19**(8): p. 1325-9.
74. Kranenburg, H.J., H.A. Hazewinkel, and B.P. Meij, *Naturally occurring spinal hyperostosis in dogs as a model for human spinal disorders.* ILAR J, 2014. **55**(1): p. 150-63.
75. Bossens, K., et al., *Diffuse idiopathic skeletal hyperostosis of the spine in a nine-year-old cat.* J Small Anim Pract, 2016. **57**(1): p. 33-5.
76. Togni, A., et al., *Radiographic and MRI characteristics of lumbar disseminated idiopathic spinal hyperostosis and spondylosis deformans in dogs.* J Small Anim Pract, 2014. **55**(7): p. 343-9.
77. Woodard, J.C., et al., *Canine diffuse idiopathic skeletal hyperostosis.* Vet Pathol, 1985. **22**(4): p. 317-26.
78. Morgan, J. and M. Stavenborn, *Disseminated idiopathic skeletal hyperostosis (DISH) in a dog.* Vet Radiol Ultrasound, 1991. **32**: p. 65-70.
79. Ghazanfar, A., et al., *Diffused idiopathic skeletal hyperostosis in a fighting Bulldog: a case report.* Iran J Vet Res, 2015. **16**(2): p. 223-5.
80. Kornmayer, M., et al., *Spinal fracture in a dog with diffuse idiopathic skeletal hyperostosis.* Vet Comp Orthop Traumatol, 2013. **26**(1): p. 76-81.
81. Oh, J., et al., *Diffuse idiopathic skeletal hyperostosis with prominent appendicular bone proliferation in a dog.* J Vet Med Sci, 2015. **77**(4): p. 493-7.

82. Ostrander, E.A., F. Galibert, and D.F. Patterson, *Canine genetics comes of age.* Trends Genet, 2000. **16**(3): p. 117-24.
83. Warraich, S., et al., *Loss of equilibrative nucleoside transporter 1 in mice leads to progressive ectopic mineralization of spinal tissues resembling diffuse idiopathic skeletal hyperostosis in humans.* J Bone Miner Res, 2013. **28**(5): p. 1135-49.
84. Hinton, D.J., et al., *Aberrant bone density in aging mice lacking the adenosine transporter ENT1.* PLoS One, 2014. **9**(2): p. e88818.
85. Bicket, A., et al., *Novel regulation of equlibrative nucleoside transporter 1 (ENT1) by receptor-stimulated Ca2+-dependent calmodulin binding.* Am J Physiol Cell Physiol, 2016. **310**(10): p. C808-20.
86. Carroll, S.H., et al., *A2B adenosine receptor promotes mesenchymal stem cell differentiation to osteoblasts and bone formation in vivo.* J Biol Chem, 2012. **287**(19): p. 15718-27.
87. Kim, J.H., et al., *Functional role of the polymorphic 647 T/C variant of ENT1 (SLC29A1) and its association with alcohol withdrawal seizures.* PLoS One, 2011. **6**(1): p. e16331.
88. Yamazaki, M., et al., *Increased type XI collagen expression in the spinal hyperostotic mouse (TWY/TWY).* Calcif. Tissue Int., 1991. **48**: p. 182-189.
89. Okawa, A., et al., *Mutation in Npps in a mouse model of ossification of the posterior longitudinal ligament of the spine.* Nat Genet, 1998. **19**(3): p. 271-3.
90. Hajjawi, M.O., et al., *Mineralisation of collagen rich soft tissues and osteocyte lacunae in Enpp1(-/-) mice.* Bone, 2014. **69**: p. 139-47.
91. Buckley, M.F., et al., *Plasma cell membrane glycoprotein PC-1. cDNA cloning of the human molecule, amino acid sequence, and chromosomal location.* J Biol Chem, 1990. **265**(29): p. 17506-11.
92. Stefan, C., S. Jansen, and M. Bollen, *NPP-type ectophosphodiesterases: unity in diversity.* Trends Biochem Sci, 2005. **30**(10): p. 542-50.
93. Kato, K., et al., *Crystal structure of Enpp1, an extracellular glycoprotein involved in bone mineralization and insulin signaling.* Proc Natl Acad Sci U S A, 2012. **109**(42): p. 16876-81.
94. Caswell, A.M., S.Y. Ali, and R.G. Russell, *Nucleoside triphosphate pyrophosphatase of rabbit matrix vesicles, a mechanism for the generation of inorganic pyrophosphate in epiphyseal cartilage.* Biochim Biophys Acta, 1987. **924**(2): p. 276-83.
95. Caswell, A.M. and R.G. Russell, *Evidence that ecto-nucleoside-triphosphate pyrophosphatase serves in the generation of extracellular inorganic pyrophosphate in human bone and articular cartilage.* Biochim Biophys Acta, 1988. **966**(3): p. 310-7.
96. Rutsch, F., et al., *Mutations in ENPP1 are associated with 'idiopathic' infantile arterial calcification.* Nat Genet, 2003. **34**(4): p. 379-81.
97. Nitschke, Y. and F. Rutsch, *Generalized arterial calcification of infancy and pseudoxanthoma elasticum: two sides of the same coin.* Front Genet, 2012. **3**: p. 302.
98. Li, Q., et al., *Mutant Enpp1asj mice as a model for generalized arterial calcification of infancy.* Dis Model Mech, 2013. **6**(5): p. 1227-35.
99. Levy-Litan, V., et al., *Autosomal-recessive hypophosphatemic rickets is associated with an inactivation mutation in the ENPP1 gene.* Am J Hum Genet, 2010. **86**(2): p. 273-8.
100. Eytan, O., et al., *Cole Disease Results from Mutations in ENPP1.* Am J Hum Genet, 2013. **93**(4): p. 752-7.
101. Okano, T., et al., *Orthotopic ossification of the spinal ligaments of Zucker fatty rats: a possible animal model for ossification of the human posterior longitudinal ligament.* J Orthop Res, 1997. **15**(6): p. 820-9.

102. Phillips, M.S., et al., *Leptin receptor missense mutation in the fatty Zucker rat.* Nat Genet, 1996. **13**(1): p. 18-9.
103. Elefteriou, F., et al., *Serum leptin level is a regulator of bone mass.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 3258-63.
104. Liu, G.Y., et al., *Leptin promotes the osteoblastic differentiation of vascular smooth muscle cells from female mice by increasing RANKL expression.* Endocrinology, 2014. **155**(2): p. 558-67.
105. Montague, C.T., et al., *Congenital leptin deficiency is associated with severe early-onset obesity in humans.* Nature, 1997. **387**(6636): p. 903-8.
106. Ho, A.M., M.D. Johnson, and D.M. Kingsley, *Role of the mouse ank gene in control of tissue calcification and arthritis.* Science, 2000. **289**(5477): p. 265-70.
107. Kim, H.J., et al., *Progressive ankylosis protein (ANK) in osteoblasts and osteoclasts controls bone formation and bone remodeling.* J Bone Miner Res, 2010. **25**(8): p. 1771-83.
108. Chen, I.P., et al., *A Phe377del mutation in ANK leads to impaired osteoblastogenesis and osteoclastogenesis in a mouse model for craniometaphyseal dysplasia (CMD).* Hum Mol Genet, 2011. **20**(5): p. 948-61.
109. Reichenberger, E., et al., *Autosomal dominant craniometaphyseal dysplasia is caused by mutations in the transmembrane protein ANK.* Am J Hum Genet, 2001. **68**(6): p. 1321-6.
110. Pendleton, A., et al., *Mutations in ANKH cause chondrocalcinosis.* Am J Hum Genet, 2002. **71**(4): p. 933-40.
111. Li, Q., et al., *The abcc6a gene expression is required for normal zebrafish development.* J Invest Dermatol, 2010. **130**(11): p. 2561-8.
112. Pomozi, V., et al., *Analysis of pseudoxanthoma elasticum-causing missense mutants of ABCC6 in vivo; pharmacological correction of the mislocalized proteins.* J Invest Dermatol, 2014. **134**(4): p. 946-53.
113. Mackay, E.W., A. Apschner, and S. Schulte-Merker, *Vitamin K reduces hypermineralisation in zebrafish models of PXE and GACI.* Development, 2015. **142**(6): p. 1095-101.
114. Apschner, A., et al., *Zebrafish enpp1 mutants exhibit pathological mineralization, mimicking features of generalized arterial calcification of infancy (GACI) and pseudoxanthoma elasticum (PXE).* Dis Model Mech, 2014. **7**(7): p. 811-22.
115. Laize, V., P. Gavaia, and M. Cancela, *Fish: a suitable system to model human bone disorders and discover drugs with osteogenic or osteotoxic activities.* Drug Discovery Today: Disease Models, 2014.
116. Rotllant, J., et al., *Calcium mobilization from fish scales is mediated by parathyroid hormone related protein via the parathyroid hormone type 1 receptor.* Regul Pept, 2005. **132**(1-3): p. 33-40.
117. Redruello, B., et al., *Isolation and characterization of piscine osteonectin and downregulation of its expression by PTH-related protein.* J Bone Miner Res, 2005. **20**(4): p. 682-92.
118. Canario, A.V., et al., *Novel bioactive parathyroid hormone and related peptides in teleost fish.* FEBS Lett, 2006. **580**(1): p. 291-9.
119. Huitema, L.F., et al., *Entpd5 is essential for skeletal mineralization and regulates phosphate homeostasis in zebrafish.* Proc Natl Acad Sci U S A, 2012. **109**(52): p. 21372-7.
120. Millan, J.L., *The role of phosphatases in the initiation of skeletal mineralization.* Calcif Tissue Int, 2013. **93**(4): p. 299-306.

121. Uitto, J., Q. Li, and Q. Jiang, *Pseudoxanthoma elasticum: molecular genetics and putative pathomechanisms.* J Invest Dermatol, 2010. **130**(3): p. 661-70.
122. Le Saux, O., et al., *The molecular and physiological roles of ABCC6: more than meets the eye.* Front Genet, 2012. **3**: p. 289.
123. Le Saux, O., et al., *Expression and in vivo rescue of human ABCC6 disease-causing mutants in mouse liver.* PLoS One, 2011. **6**(9): p. e24738.
124. Beck, K., et al., *Analysis of ABCC6 (MRP6) in normal human tissues.* Histochem Cell Biol, 2005. **123**(4-5): p. 517-28.
125. Bergen, A.A., et al., *Mutations in ABCC6 cause pseudoxanthoma elasticum.* Nat Genet, 2000. **25**(2): p. 228-31.
126. Le Saux, O., et al., *Mutations in a gene encoding an ABC transporter cause pseudoxanthoma elasticum.* Nat Genet, 2000. **25**(2): p. 223-7.
127. Ringpfeil, F., et al., *Pseudoxanthoma elasticum: mutations in the MRP6 gene encoding a transmembrane ATP-binding cassette (ABC) transporter.* Proc Natl Acad Sci U S A, 2000. **97**(11): p. 6001-6.
128. Klement, J.F., et al., *Targeted ablation of the abcc6 gene results in ectopic mineralization of connective tissues.* Mol Cell Biol, 2005. **25**(18): p. 8299-310.
129. Jansen, R.S., et al., *ABCC6 prevents ectopic mineralization seen in pseudoxanthoma elasticum by inducing cellular nucleotide release.* Proc Natl Acad Sci U S A, 2013. **110**(50): p. 20206-11.
130. Dabisch-Ruthe, M., et al., *Variants in genes encoding pyrophosphate metabolizing enzymes are associated with Pseudoxanthoma elasticum.* Clin Biochem, 2014. **47**(15): p. 60-7.
131. Brewerton, D.A., et al., *Ankylosing spondylitis and HLA-B27.* Lancet, 1973. **1**: p. 904-907.
132. Schlosstein, L., et al., *High association of an HL-A antigen, W27, with ankylosing spondylitis.* N Engl J Med, 1973. **288**(14): p. 704-6.
133. Shapiro, R.F., et al., *The association of HL-A B27 with Forestier's disease (vertebral ankylosing hyperostosis).* J Rheumatol., 1976 **3**(1): p. 4-8.
134. Shapiro, R.F., et al., *HLA-B27 and modified bone formation.* Lancet, 1976 **1**(7953): p. 230-1.
135. Ercilla, M.G., et al., *HLA antigens in Forestier's disease, ankylosing spondylitis, and polyarthrosis of the hands.* J Rheumatol Suppl, 1977. **3**: p. 89-93.
136. Rosenthal, M., I. Bahous, and W. Müller, *Letter: HL-A B27 and modified bone formation.* Lancet, 1976 **1**(7958): p. 543.
137. Rotés Querol, J. and G. Ercilla González, *Letter: HLA-B27 and modified bone formation.* Lancet, 1976 **1**(7957): p. 482-3.
138. Brigode, M. and R.J. Francois, *Histocompatibility antigens in vertebral ankylosing hyperostosis.* J Rheumatol., 1977 **4**(4): p. 429-34.
139. Spagnola, A.M., P.H. Bennett, and P.I. Terasaki, *Vertebral ankylosing hyperostosis (Forestier's disease) and HLA antigens in Pima Indians.* Arthritis Rheum., 1978 **21**(4): p. 467-72.
140. Perry, J.D., et al., *Ankylosing hyperostosis: a study of HLA A, B, and C antigens.* Ann Rheum Dis., 1979 **38**(1): p. 72-3.
141. Dostál, C., et al., *HLA antigens in vertebral ankylosing hyperostosis (Forestier's disease).* J Hyg Epidemiol Microbiol Immunol., 1983. **27**(1): p. 98-102.
142. Rosenthal, M., I. Bahous, and W. Muller, *Increased frequency of HLA B8 in hyperostotic spondylosis.* J Rheumatol Suppl., 1977. **3**: p. 94-6.

143. Ercilla González, G., M.A. Brancós, and Y. Breisse, *Histocompatability antigens in Forestier's disease, polyarthrosis and ankylosing spondylitis*, in *HLA and Disease*. 1976, INSERM: Paris. p. 28.
144. Brown, M.A., et al., *Genetic control of bone density and turnover: role of the collagen 1alpha1, estrogen receptor, and vitamin D receptor genes.* J Bone Miner Res, 2001. **16**(4): p. 758-64.
145. Havelka, S., et al., *Collagen type I(alpha 1) and vitamin D receptor polymorphisms in diffuse idiopathic skeletal hyperostosis.* Clin Rheumatol., 2002 **21**(4): p. 347-8.
146. Tanaka, T., et al., *Genomewide linkage and linkage disequilibrium analyses identify COL6A1, on chromosome 21, as the locus for ossification of the posterior longitudinal ligament of the spine.* Am J Hum Genet, 2003. **73**(4): p. 812-22.
147. Jun, J.K. and S.M. Kim, *Association study of fibroblast growth factor 2 and fibroblast growth factor receptors gene polymorphism in korean ossification of the posterior longitudinal ligament patients.* J Korean Neurosurg Soc, 2012. **52**(1): p. 7-13.
148. Marie, P.J., *Fibroblast growth factor signaling controlling osteoblast differentiation.* Gene, 2003. **316**: p. 23-32.
149. Yamaguchi, M., *[Genetic study on OPLL in the cervical spine with HLA haplotype]*. Nihon Seikeigeka Gakkai Zasshi, 1991. **65**(8): p. 527-35.
150. Matsunaga, S., et al., *Genetic analysis of ossification of the posterior longitudinal ligament.* Spine (Phila Pa 1976), 1999. **24**(10): p. 937-8; discussion 939.
151. Sakou, T., et al., *Genetic study of ossification of the posterior longitudinal ligament in the cervical spine with human leukocyte antigen haplotype.* Spine (Phila Pa 1976), 1991. **16**(11): p. 1249-52.
152. Stetler, W.R., F. La Marca, and P. Park, *The genetics of ossification of the posterior longitudinal ligament.* Neurosurg Focus, 2011. **30**(3): p. E7.
153. Ogata, N., et al., *Association of bone metabolism regulatory factor gene polymorphisms with susceptibility to ossification of the posterior longitudinal ligament of the spine and its severity.* Spine (Phila Pa 1976), 2002. **27**(16): p. 1765-71.
154. Horikoshi, T., et al., *A large-scale genetic association study of ossification of the posterior longitudinal ligament of the spine.* Hum Genet, 2006. **119**(6): p. 611-6.
155. Kim, D.H., et al., *The Insertion/Deletion Polymorphism of Angiotensin I Converting Enzyme Gene is Associated With Ossification of the Posterior Longitudinal Ligament in the Korean Population.* Ann Rehabil Med, 2014. **38**(1): p. 1-5.
156. Wang, H., et al., *Association between two polymorphisms of the bone morpho-genetic protein-2 gene with genetic susceptibility to ossification of the posterior longitudinal ligament of the cervical spine and its severity.* Chin Med J (Engl), 2008. **121**(18): p. 1806-10.
157. Wang, H., et al., *Association of bone morphogenetic protein-2 gene polymorphisms with susceptibility to ossification of the posterior longitudinal ligament of the spine and its severity in Chinese patients.* Eur Spine J, 2008. **17**(7): p. 956-64.
158. Yan, L., et al., *A single nucleotide polymorphism in the human bone morphogenetic protein-2 gene (109T > G) affects the Smad signaling pathway and the predisposition to ossification of the posterior longitudinal ligament of the spine.* Chin Med J (Engl), 2013. **126**(6): p. 1112-8.
159. Furushima, K., et al., *Large-scale screening for candidate genes of ossification of the posterior longitudinal ligament of the spine.* J Bone Miner Res, 2002. **17**(1): p. 128-37.
160. Meng, X.L., et al., *T allele at site 6007 of bone morphogenetic protein-4 gene increases genetic susceptibility to ossification of the posterior longitudinal ligament in male Chinese Han population.* Chin Med J (Engl), 2010. **123**(18): p. 2537-42.

161. Ren, Y., et al., *A new haplotype in BMP4 implicated in ossification of the posterior longitudinal ligament (OPLL) in a Chinese population.* J Orthop Res, 2012. **30**(5): p. 748-56.
162. Ren, Y., et al., *Association of a BMP9 haplotype with ossification of the posterior longitudinal ligament (OPLL) in a Chinese population.* PLoS One, 2012. **7**(7): p. e40587.
163. Wei, W., et al., *Whole exome sequencing implicates PTCH1 and COL17A1 genes in ossification of the posterior longitudinal ligament of the cervical spine in Chinese patients.* Genet Mol Res, 2014. **13**(1): p. 1794-804.
164. Nakamura, I., et al., *Association of the human NPPS gene with ossification of the posterior longitudinal ligament of the spine (OPLL).* Hum Genet, 1999. **104**(6): p. 492-7.
165. Koshizuka, Y., et al., *Nucleotide pyrophosphatase gene polymorphism associated with ossification of the posterior longitudinal ligament of the spine.* J Bone Miner Res, 2002. **17**(1): p. 138-44.
166. He, Z., et al., *Association of NPP1 polymorphism with postoperative progression of ossification of the posterior longitudinal ligament in Chinese patients.* Genet Mol Res, 2013. **12**(4): p. 4648-55.
167. Guo, Q., et al., *Association between single nucleotide polymorphism of IL15RA gene with susceptibility to ossification of the posterior longitudinal ligament of the spine.* J Orthop Surg Res, 2014. **9**: p. 103.
168. Kim, D.H., et al., *Association between interleukin 15 receptor, alpha (IL15RA) polymorphism and Korean patients with ossification of the posterior longitudinal ligament.* Cytokine, 2011. **55**(3): p. 343-6.
169. Numasawa, T., et al., *Human retinoic X receptor beta: complete genomic sequence and mutation search for ossification of posterior longitudinal ligament of the spine.* J Bone Miner Res, 1999. **14**(4): p. 500-8.
170. Kamiya, M., et al., *Association between a polymorphism of the transforming growth factor-beta1 gene and genetic susceptibility to ossification of the posterior longitudinal ligament in Japanese patients.* Spine (Phila Pa 1976), 2001. **26**(11): p. 1264-6; discussion 1266-7.
171. Kobashi, G., et al., *FokI variant of vitamin D receptor gene and factors related to atherosclerosis associated with ossification of the posterior longitudinal ligament of the spine: a multi-hospital case-control study.* Spine (Phila Pa 1976), 2008. **33**(16): p. E553-8.
172. Nakajima, M., et al., *A genome-wide association study identifies susceptibility loci for ossification of the posterior longitudinal ligament of the spine.* Nat Genet, 2014. **46**(9): p. 1012-6.
173. Chon, J., et al., *Association between BH3 interacting domain death agonist (BID) gene polymorphism and ossification of the posterior longitudinal ligament in Korean population.* Mol Biol Rep, 2014. **41**(2): p. 895-9.
174. Jekarl, D.W., et al., *TGFBR2 gene polymorphism is associated with ossification of the posterior longitudinal ligament.* J Clin Neurosci, 2013. **20**(3): p. 453-6.
175. Chin, D.K., et al., *Association of VKORC1-1639G>A polymorphism with susceptibility to ossification of the posterior longitudinal ligament of the spine: a Korean study.* Acta Neurochir (Wien), 2013. **155**(10): p. 1937-42.
176. Kim, K.T., et al., *Association of interferon gamma polymorphism with ossification of the posterior longitudinal ligament in the korean population.* Immunol Invest, 2012. **41**(8): p. 876-87.

177. Maeda, S., et al., *Functional impact of human collagen alpha2(XI) gene polymorphism in pathogenesis of ossification of the posterior longitudinal ligament of the spine.* J Bone Miner Res, 2001. **16**(5): p. 948-57.
178. Goding, J.W., B. Grobben, and H. Slegers, *Physiological and pathophysiological functions of the ecto-nucleotide pyrophosphatase/phosphodiesterase family.* Biochim Biophys Acta, 2003. **1638**(1): p. 1-19.
179. Meyre, D., et al., *Variants of ENPP1 are associated with childhood and adult obesity and increase the risk of glucose intolerance and type 2 diabetes.* Nat Genet, 2005. **37**(8): p. 863-7.
180. Tahara, M., et al., *The extent of ossification of posterior longitudinal ligament of the spine associated with nucleotide pyrophosphatase gene and leptin receptor gene polymorphisms.* Spine (Phila Pa 1976), 2005. **30**(8): p. 877-80; discussion 881.
181. Chen, X., et al., *Targeted next-generation sequencing reveals multiple deleterious variants in OPLL-associated genes.* Sci Rep, 2016. **6**: p. 26962.
182. Wrana, J.L. and L. Attisano, *The Smad pathway.* Cytokine Growth Factor Rev, 2000. **11**(1-2): p. 5-13.
183. Golub, E. and K. Boesze- Battaglia, *The role of alkaline phosphatase in mineralization.* Curr Opin in Orthop, 2007. **18**: p. 444-448.
184. Han, I.B., et al., *Association of transforming growth factor-beta 1 gene polymorphism with genetic susceptibility to ossification of the posterior longitudinal ligament in Korean patients.* Genet Mol Res, 2013. **12**(4): p. 4807-16.
185. Kawaguchi, Y., et al., *Association between polymorphism of the transforming growth factor-beta1 gene with the radiologic characteristic and ossification of the posterior longitudinal ligament.* Spine (Phila Pa 1976), 2003. **28**(13): p. 1424-6.
186. Barry, F., et al., *Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components.* Exp Cell Res, 2001. **268**(2): p. 189-200.
187. Karasugi, T., et al., *A genome-wide sib-pair linkage analysis of ossification of the posterior longitudinal ligament of the spine.* J Bone Miner Metab, 2013. **31**(2): p. 136-43.
188. Nobta, M., et al., *Critical regulation of bone morphogenetic protein-induced osteoblastic differentiation by Delta1/Jagged1-activated Notch1 signaling.* J Biol Chem, 2005. **280**(16): p. 15842-8.
189. Aquaron, R., *Alkaptonuria: a very rare metabolic disorder.* Indian J Biochem Biophys, 2013. **50**(5): p. 339-44.
190. Consortium., A., *Autosomal dominant hypophosphataemic rickets is associated with mutations in FGF23.* Nat Genet., 2000 **26**(3): p. 345-8.
191. Prie, D., et al., *Nephrolithiasis and osteoporosis associated with hypophosphatemia caused by mutations in the type 2a sodium-phosphate cotransporter.* N Engl J Med, 2002. **347**(13): p. 983-91.
192. Lu, Y., et al., *The biological function of DMP-1 in osteocyte maturation is mediated by its 57-kDa C-terminal fragment.* J Bone Miner Res, 2011. **26**(2): p. 331-40.
193. Martin, A., et al., *Degradation of MEPE, DMP1, and release of SIBLING ASARM-peptides (minhibins): ASARM-peptide(s) are directly responsible for defective mineralization in HYP.* Endocrinology, 2008. **149**(4): p. 1757-72.
194. Lin, Z., et al., *Chloride channel (Clc)-5 is necessary for exocytic trafficking of Na+/H+ exchanger 3 (NHE3).* J Biol Chem, 2011. **286**(26): p. 22833-45.
195. Saito, T., et al., *A patient with hypophosphatemic rickets and ossification of posterior longitudinal ligament caused by a novel homozygous mutation in ENPP1 gene.* Bone, 2011. **49**(4): p. 913-6.

196. McKiernan, F.E., R.L. Berg, and J. Fuehrer, *Clinical and radiographic findings in adults with persistent hypophosphatasemia.* J Bone Miner Res, 2014. **29**(7): p. 1651-60.
197. Mak, A. and C.C. Mok, *Diffuse skeletal hyperostosis and pseudohypoparathyroidism.* Rheumatology (Oxford), 2005. **44**(2): p. 182.
198. John, D.R. and P.P. Suthar, *Radiological Features of Long-Standing Hypoparathyroidism.* Pol J Radiol, 2016. **81**: p. 42-5.
199. Unverdi, S., et al., *Idiopathic hypoparathyroidism mimicking diffuse idiopathic skeletal hyperostosis.* J Clin Rheumatol, 2009. **15**(7): p. 361-2.
200. Lambert, R.G. and E.J. Becker, *Diffuse skeletal hyperostosis in idiopathic hypoparathyroidism.* Clin Radiol, 1989. **40**(2): p. 212-5.
201. Rathore, F.A., S.B. Ayaz, and S.N. Mansoor, *Ochronotic Arthropathy: Two Case Reports from a Developing Country.* Clin Med Insights Arthritis Musculoskelet Disord, 2016. **9**: p. 15-20.
202. Schmidt, R.F., I.M. Goldstein, and J.K. Liu, *Ossified ligamentum flavum causing spinal cord compression in a patient with acromegaly.* J Clin Neurosci, 2013. **20**(11): p. 1599-603.
203. Rivas, A.M. and J. Lado-Abeal, *Diffuse idiopathic skeletal hyperostosis and familial hypocalciuric hypercalcemia: a unique association in a young female.* Am J Med Sci, 2013. **346**(3): p. 247-9.
204. Elli, F.M., et al., *The Complex GNAS Imprinted Locus and Mesenchymal Stem Cells Differentiation.* Horm Metab Res, 2016.
205. Gunther, T., et al., *Genetic ablation of parathyroid glands reveals another source of parathyroid hormone.* Nature, 2000. **406**(6792): p. 199-203.
206. Blaine, J., M. Chonchol, and M. Levi, *Correction. Renal control of calcium, phosphate, and magnesium homeostasis.* Clin J Am Soc Nephrol, 2015. **10**(10): p. 1886-7.
207. Ben-Shlomo, A. and S. Melmed, *Acromegaly.* Endocrinol Metab Clin North Am, 2008. **37**(1): p. 101-22, viii.
208. Reinecke, M., et al., *Effect of growth hormone and insulin-like growth factor I (IGF-I) on the expression of IGF-I messenger ribonucleic acid and peptide in rat tibial growth plate and articular chondrocytes in vivo.* Endocrinology, 2000. **141**(8): p. 2847-53.
209. Altomonte, L., et al., *Growth hormone secretion in diffuse idiopathic skeletal hyperostosis.* Ann Ital Med Int, 1992. **7**(1): p. 30-3.
210. Rivas, A.M. and J. Lado-Abeal, *Diffuse idiopathic skeletal hyperostosis and familial hypocalciuric hypercalcemia: a unique association in a young female.* Am J Med Sci., 2013 **346**(3): p. 247-9.
211. Pappone, N., et al., *Is diffuse idiopathic skeletal hyperostosis a disease or a syndrome? Need for studies on angiogenesis-stimulating activity.* Rheumatology (Oxford), 2016.
212. Akune, T., et al., *Insulin secretory response is positively associated with the extent of ossification of the posterior longitudinal ligament of the spine.* J Bone Joint Surg Am, 2001. **83-A**(10): p. 1537-44.
213. Mader, R. and I. Lavi, *Diabetes mellitus and hypertension as risk factors for early diffuse idiopathic skeletal hyperostosis (DISH).* Osteoarthritis Cartilage, 2009. **17**(6): p. 825-8.
214. Mader, R., et al., *Metabolic syndrome and cardiovascular risk in patients with diffuse idiopathic skeletal hyperostosis.* Semin Arthritis Rheum, 2009. **38**(5): p. 361-5.

215. Julkunen, H., O.P. Heinonen, and K. Pyorala, *Hyperostosis of the spine in an adult population. Its relation to hyperglycaemia and obesity.* Ann Rheum Dis, 1971. **30**(6): p. 605-12.
216. Kaur, J., *A comprehensive review on metabolic syndrome.* Cardiol Res Pract, 2014. **2014**: p. 943162.
217. Pavelkova, A. and K. Pavelka, *[Diffuse idiopathic skeletal hyperostosis and its relation to metabolic parameters]*. Vnitr Lek, 2006. **52**(5): p. 477-80.
218. Huvenne, H., et al., *Rare Genetic Forms of Obesity: Clinical Approach and Current Treatments in 2016.* Obes Facts, 2016. **9**(3): p. 158-73.
219. Abate, N., et al., *ENPP1/PC-1 K121Q polymorphism and genetic susceptibility to type 2 diabetes.* Diabetes, 2005. **54**(4): p. 1207-13.
220. Lin, C.C., C.T. Wu, and L.S. Wu, *Ectonucleotide pyrophosphatase/phosphodiesterase 1 K173Q polymorphism is associated with diabetic nephropathy in the Taiwanese population.* Genet Test Mol Biomarkers, 2011. **15**(4): p. 239-42.
221. Forrestier, J. and R.-Q. J, *Senile ankylosing hyperostosis of the spine.* Ann Rheum Dis, 1950. **9**: p. 321-30.
222. Onishi, E., H. Sano, and M. Matsushita, *Surgical Treatment for Thoracic Myelopathy Due to Simultaneous Ossification of the Posterior Longitudinal Ligament and Ligamentum Flavum at the Same Level.* Clin Spine Surg, 2016.
223. Li, M., et al., *Management of thoracic myelopathy caused by ossification of the posterior longitudinal ligament combined with ossification of the ligamentum flavum-a retrospective study.* Spine J, 2012. **12**(12): p. 1093-102.
224. Mazières, B., M. Jung-Rozenfarb, and J. Arlet, *[Paget's disease, ankylosing vertebral hyperostosis and hyperostosis frontalis interna]*. Sem Hop., 1978 **54**(13-16): p. 521-5.
225. Morales, A.A., et al., *Coexistence of Paget's bone disease and diffuse idiopathic skeletal hyperostosis in males.* Clin Exp Rheumatol., 1993 **11**(4): p. 361-5.
226. Resnick, D., et al., *Radiographic abnormalities of rheumatoid arthritis in patients with diffuse idiopathic skeletal hyperostosis.* Arthritis Rheum., 1978 **21**(1): p. 1-5.
227. Forster, T.J., J.C. Leek, and D.L. Robbins, *Peripheral hyperostosis in rheumatoid arthritis in the absence of axial involvement.* J Rheumatol., 1981 **8**(4): p. 653-7.
228. Mata, S., et al., *Absence of an association of rheumatoid arthritis and diffuse idiopathic skeletal hyperostosis: a case-control study.* J Rheumatol., 1995 **22**(11): p. 2062-4.
229. Littlejohn, G.O. and S. Hall, *Diffuse idiopathic skeletal hyperostosis and new bone formation in male gouty subjects. A radiologic study.* Rheumatol Int., 1982. **2**(2): p. 83-6.
230. Constantz, R. and B. R., *Case report 237: gout associated with diffuse idiopathic skeletal hyperostosis (DISH).* Skeletal Radiol., 1983. **10**(2): p. 117-20.
231. Kim, T.J., et al., *Prevalence of ossification of posterior longitudinal ligament in patients with ankylosing spondylitis.* J Rheumatol, 2007. **34**(12): p. 2460-2.
232. Cortes, A., et al., *Identification of multiple risk variants for ankylosing spondylitis through high-density genotyping of immune-related loci.* Nat Genet, 2013. **45**(7): p. 730-8.
233. Reveille, J.D., *Genetics of spondyloarthritis--beyond the MHC.* Nat Rev Rheumatol, 2012. **8**(5): p. 296-304.
234. Robinson, P.C. and M.A. Brown, *The genetics of ankylosing spondylitis and axial spondyloarthritis.* Rheum Dis Clin North Am, 2012. **38**(3): p. 539-53.
235. Brown, M.A., et al., *HLA class I associations of ankylosing spondylitis in the white population in the United Kingdom.* Ann Rheum Dis, 1996. **55**(4): p. 268-70.

236. Brown, M.A., et al., *Susceptibility to ankylosing spondylitis in twins: the role of genes, HLA, and the environment.* Arthritis Rheum, 1997. **40**(10): p. 1823-8.
237. Moon, K.H. and Y.T. Kim, *Medical Treatment of Ankylosing Spondylitis.* Hip Pelvis, 2014. **26**(3): p. 129-35.
238. Cortes, A., et al., *Major histocompatibility complex associations of ankylosing spondylitis are complex and involve further epistasis with ERAP1.* Nat Commun, 2015. **6**: p. 7146.
239. Kadi, A., et al., *Investigating the genetic association between ERAP1 and spondyloarthritis.* Ann Rheum Dis, 2013. **72**(4): p. 608-13.
240. Bettencourt, B.F., et al., *Protective effect of an ERAP1 haplotype in ankylosing spondylitis: investigating non-MHC genes in HLA-B27-positive individuals.* Rheumatology (Oxford), 2013. **52**(12): p. 2168-76.
241. Timms, A.E., et al., *The interleukin 1 gene cluster contains a major susceptibility locus for ankylosing spondylitis.* Am J Hum Genet, 2004. **75**(4): p. 587-95.
242. Smith, J.A. and R.A. Colbert, *Review: The interleukin-23/interleukin-17 axis in spondyloarthritis pathogenesis: Th17 and beyond.* Arthritis Rheumatol, 2014. **66**(2): p. 231-41.
243. Reveille, J.D., et al., *Genome-wide association study of ankylosing spondylitis identifies non-MHC susceptibility loci.* Nat Genet, 2010. **42**(2): p. 123-7.
244. Lin, Z., et al., *A genome-wide association study in Han Chinese identifies new susceptibility loci for ankylosing spondylitis.* Nat Genet, 2011. **44**(1): p. 73-7.
245. Urano, T., et al., *Single-nucleotide polymorphism in the hyaluronan and proteoglycan link protein 1 (HAPLN1) gene is associated with spinal osteophyte formation and disc degeneration in Japanese women.* Eur Spine J, 2011. **20**(4): p. 572-7.
246. Takai, A., et al., *Anterior neural development requires Del1, a matrix-associated protein that attenuates canonical Wnt signaling via the Ror2 pathway.* Development, 2010. **137**(19): p. 3293-302.
247. Ousingsawat, J., et al., *Anoctamin-6 controls bone mineralization by activating the calcium transporter NCX1*. J Biol Chem, 2015. **290**(10): p. 6270-80.
248. Tsui, H.W., et al., *ANKH variants associated with ankylosing spondylitis: gender differences.* Arthritis Res Ther, 2005. **7**(3): p. R513-25.
249. Stella, J., et al., *Effects of Different Variants in the ENPP1 Gene on the Functional Properties of Ectonucleotide Pyrophosphatase/Phosphodiesterase Family Member 1*. Hum Mutat, 2016. **37**(11): p. 1190-1201.
250. Santoro, N., et al., *Effect of the rs997509 polymorphism on the association between ectonucleotide pyrophosphatase phosphodiesterase 1 and metabolic syndrome and impaired glucose tolerance in childhood obesity.* J Clin Endocrinol Metab, 2009. **94**(1): p. 300-5.
251. Zhang, Y., et al., *Investigation of the role of ENPP1 and TNAP genes in chondrocalcinosis.* Rheumatology (Oxford), 2007. **46**(4): p. 586-9.
252. Couto, A.R., et al., *Investigating ANKH and ENPP1 in Slovakian families with chondrocalcinosis.* Rheumatol Int, 2012. **32**(9): p. 2745-51.
253. Ho, H.H., W.E. Yang, and S.F. Luo, *Ankylosing spondylitis with severe chondrocalcinosis: case report.* Changgeng Yi Xue Za Zhi, 1999. **22**(3): p. 536-40.
254. Gascon, A., J.C. Cobeta-Garcia, and E. Iglesias, *Hypomagnesemia and chondrocalcinosis in Gitelman syndrome.* Am J Med, 1999. **107**(3): p. 301-2.
255. Smith, R. and P. Wordsworth, *Clinical and Biochemical Disorders of the Skeleton*. 1st ed. 2005, Oxford: Oxford University Press. 471.

256. Resnick, D., et al., *Clinical, radiographic and pathologic abnormalities in calcium pyrophosphate dihydrate deposition disease (CPPD): pseudogout.* Radiology, 1977. **122**(1): p. 1-15.
257. Resnick, D. and C. Pineda, *Vertebral involvement in calcium pyrophosphate dihydrate crystal deposition disease. Radiographic-pathological correlation.* Radiology, 1984. **153**(1): p. 55-60.
258. Muthukumar, N., U. Karuppaswamy, and B. Sankarasubbu, *Calcium pyrophosphate dihydrate deposition disease causing thoracic cord compression: case report.* Neurosurgery, 2000. **46**(1): p. 222-5.
259. Kennett, S. and H. Pollick, *Jaw lesions in familial hyperparathyroidism.* Oral Surg Oral Med Oral Pathol, 1971. **31**(4): p. 502-10.
260. Adamson, T.C., 3rd, et al., *Hand and wrist arthropathies of hemochromatosis and calcium pyrophosphate deposition disease: distinct radiographic features.* Radiology, 1983. **147**(2): p. 377-81.
261. Kockara, A.S., et al., *Gitelman's syndrome associated with chondrocalcinosis: a case report.* Ren Fail, 2013. **35**(9): p. 1285-8.
262. Zitnan, D. and S. Sitaj, *Chondrocalcinosis Articularis: Section V. Summary.* Ann Rheum Dis, 1963. **22**(3): p. 167-70.
263. Couto, A.R. and M.A. Brown, *Genetic factors in the pathogenesis of CPPD crystal deposition disease.* Curr Rheumatol Rep, 2007. **9**(3): p. 231-6.
264. Bjelle, A., U. Edvinsson, and A. Hagstam, *Pyrophosphate arthropathy in two Swedish families.* Arthritis Rheum, 1982. **25**(1): p. 66-74.
265. Rodriguez-Valverde, V., et al., *Hereditary articular chondrocalcinosis. Clinical and genetic features in 13 pedigrees.* Am J Med, 1988. **84**(1): p. 101-6.
266. Baldwin, C.T., et al., *Linkage of early-onset osteoarthritis and chondrocalcinosis to human chromosome 8q.* Am J Hum Genet, 1995. **56**(3): p. 692-7.
267. Andrew, L.J., et al., *Refinement of the chromosome 5p locus for familial calcium pyrophosphate dihydrate deposition disease.* Am J Hum Genet, 1999. **64**(1): p. 136-45.
268. Zhang, Y., et al., *Association of sporadic chondrocalcinosis with a -4-basepair G-to-A transition in the 5'-untranslated region of ANKH that promotes enhanced expression of ANKH protein and excess generation of extracellular inorganic pyrophosphate.* Arthritis Rheum, 2005. **52**(4): p. 1110-7.
269. Netter, P., et al., *The ANKH gene and familial calcium pyrophosphate dihydrate deposition disease.* Joint Bone Spine, 2004. **71**(5): p. 365-8.
270. Firestein, G.S., *Evolving concepts of rheumatoid arthritis.* Nature, 2003. **423**(6937): p. 356-61.
271. Phay, J.E., H.B. Hussain, and J.F. Moley, *Cloning and expression analysis of a novel member of the facilitative glucose transporter family, SLC2A9 (GLUT9).* Genomics, 2000. **66**(2): p. 217-20.
272. Anzai, N., et al., *Plasma urate level is directly regulated by a voltage-driven urate efflux transporter URATv1 (SLC2A9) in humans.* J Biol Chem, 2008. **283**(40): p. 26834-8.
273. Jutabha, P., et al., *Human sodium phosphate transporter 4 (hNPT4/SLC17A3) as a common renal secretory pathway for drugs and urate.* J Biol Chem, 2010. **285**(45): p. 35123-32.
274. Basseville, A. and S.E. Bates, *Gout, genetics and ABC transporters.* F1000 Biol Rep, 2011. **3**: p. 23.

275. Matsuo, H., et al., *Common defects of ABCG2, a high-capacity urate exporter, cause gout: a function-based genetic analysis in a Japanese population.* Sci Transl Med, 2009. **1**(5): p. 5ra11.
276. Roberts, J. and T.A. Burch, *Osteoarthritis prevalence in adults by age, sex, race, and geographic area.* Vital Health Stat 11, 1966(15): p. 1-27.
277. Stefansson, S.E., et al., *Genomewide scan for hand osteoarthritis: a novel mutation in matrilin-3.* Am J Hum Genet, 2003. **72**(6): p. 1448-59.
278. Loughlin, J., et al., *Functional variants within the secreted frizzled-related protein 3 gene are associated with hip osteoarthritis in females.* Proc Natl Acad Sci U S A, 2004. **101**(26): p. 9757-62.
279. Wagener, R., B. Kobbe, and M. Paulsson, *Primary structure of matrilin-3, a new member of a family of extracellular matrix proteins related to cartilage matrix protein (matrilin-1) and von Willebrand factor.* FEBS Lett, 1997. **413**(1): p. 129-34.
280. Fresquet, M., et al., *Structural and functional characterization of recombinant matrilin-3 A-domain and implications for human genetic bone diseases.* J Biol Chem, 2007. **282**(48): p. 34634-43.
281. Pei, M., J. Luo, and Q. Chen, *Enhancing and maintaining chondrogenesis of synovial fibroblasts by cartilage extracellular matrix protein matrilins.* Osteoarthritis Cartilage, 2008. **16**(9): p. 1110-7.
282. Pullig, O., et al., *MATN3 (matrilin-3) sequence variation (pT303M) is a risk factor for osteoarthritis of the CMC1 joint of the hand, but not for knee osteoarthritis.* Ann Rheum Dis, 2007. **66**(2): p. 279-80.
283. Mostert, A.K., et al., *Familial multiple epiphyseal dysplasia due to a matrilin-3 mutation: further delineation of the phenotype including 40 years follow-up.* Am J Med Genet A, 2003. **120A**(4): p. 490-7.
284. Jiang, Q., et al., *Replication of the association of the aspartic acid repeat polymorphism in the asporin gene with knee-osteoarthritis susceptibility in Han Chinese.* J Hum Genet, 2006. **51**(12): p. 1068-72.
285. Arellano-Perez-Vertti, R.D., et al., *[D-repeat polymorphism in the ASPN gene in knee osteoarthritis in females in Torreon, Coahuila. Case-control study].* Acta Ortop Mex, 2014. **28**(6): p. 363-8.
286. Song, Y.Q., et al., *Association of the asporin D14 allele with lumbar-disc degeneration in Asians.* Am J Hum Genet, 2008. **82**(3): p. 744-7.
287. Liu, D., et al., *Association of an asporin repeat polymorphism with ankylosing spondylitis in Han Chinese population: a case-control study.* Clin Invest Med, 2010. **33**(1): p. E63-8.
288. Storm, E.E., et al., *Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily.* Nature, 1994. **368**(6472): p. 639-43.
289. Francis-West, P.H., et al., *Mechanisms of GDF-5 action during skeletal development.* Development, 1999. **126**(6): p. 1305-15.
290. Evangelou, E., et al., *Large-scale analysis of association between GDF5 and FRZB variants and osteoarthritis of the hip, knee, and hand.* Arthritis Rheum, 2009. **60**(6): p. 1710-21.
291. Umair, M., et al., *Novel Homozygous Sequence Variants in the GDF5 Gene Underlie Acromesomelic Dysplasia type-Grebe (AMDG) in Consanguineous Families.* Congenit Anom (Kyoto), 2016.
292. Thomas, J.T., et al., *Disruption of human limb morphogenesis by a dominant negative mutation in CDMP1.* Nat Genet, 1997. **17**(1): p. 58-64.

293. Knowlton, R.G., et al., *Genetic linkage of a polymorphism in the type II procollagen gene (COL2A1) to primary osteoarthritis associated with mild chondrodysplasia.* N Engl J Med, 1990. **322**(8): p. 526-30.
294. Meulenbelt, I., et al., *Strong linkage on 2q33.3 to familial early-onset generalized osteoarthritis and a consideration of two positional candidate genes.* Eur J Hum Genet, 2006. **14**(12): p. 1280-7.
295. Meulenbelt, I., et al., *Large replication study and meta-analyses of DVWA as an osteoarthritis susceptibility locus in European and Asian populations.* Hum Mol Genet, 2009. **18**(8): p. 1518-23.
296. Miyamoto, Y., et al., *Common variants in DVWA on chromosome 3p24.3 are associated with susceptibility to knee osteoarthritis.* Nat Genet, 2008. **40**(8): p. 994-8.
297. Lalam, R.K., V.N. Cassar-Pullicino, and N. Winn, *Paget Disease of Bone.* Semin Musculoskelet Radiol, 2016. **20**(3): p. 287-299.
298. Dell'Atti, C., et al., *The spine in Paget's disease.* Skeletal Radiol, 2007. **36**(7): p. 609-26.
299. Martin, T.J., *Paracrine regulation of osteoclast formation and activity: milestones in discovery.* J Musculoskelet Neuronal Interact, 2004. **4**(3): p. 243-53.
300. Arko, B., et al., *Sequence variations in the osteoprotegerin gene promoter in patients with postmenopausal osteoporosis.* J Clin Endocrinol Metab, 2002. **87**(9): p. 4080-4.
301. Ramos, Y.F., et al., *A gain of function mutation in TNFRSF11B encoding osteoprotegerin causes osteoarthritis with chondrocalcinosis.* Ann Rheum Dis, 2015. **74**(9): p. 1756-62.
302. Divisato, G., et al., *ZNF687 Mutations in Severe Paget Disease of Bone Associated with Giant Cell Tumor.* Am J Hum Genet, 2016. **98**(2): p. 275-86.
303. Malovannaya, A., et al., *Analysis of the human endogenous coregulator complexome.* Cell, 2011. **145**(5): p. 787-99.
304. nº12/2005, L., *"Informação genética e Informação de Saúde"*, D.s.I.-A.d.J.d. 2006, Editor. 2005. p. 606-611.
305. van der Linden, S., H.A. Valkenburg, and A. Cats, *Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria.* Arthritis Rheum, 1984. **27**(4): p. 361-8.
306. Miller, S.A., D.D. Dykes, and H.F. Polesky, *A simple salting out procedure for extracting DNA from human nucleated cells.* Nucleic Acids Res, 1988. **16**(3): p. 1215.
307. Hellemans, J., et al., *Loss-of-function mutations in LEMD3 result in osteopoikilosis, Buschke-Ollendorff syndrome and melorheostosis.* Nat Genet, 2004. **36**(11): p. 1213-8.
308. Miksch, S., et al., *Molecular genetics of pseudoxanthoma elasticum: type and frequency of mutations in ABCC6.* Hum Mutat, 2005. **26**(3): p. 235-48.
309. Cai, L., et al., *A novel Q378X mutation exists in the transmembrane transporter protein ABCC6 and its pseudogene: implications for mutation analysis in pseudoxanthoma elasticum.* J Mol Med (Berl), 2001. **79**(9): p. 536-46.
310. Chomczynski, P. and N. Sacchi, *Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.* Anal Biochem, 1987. **162**(1): p. 156-9.
311. Glaudemans, B., et al., *Novel NCC mutants and functional analysis in a new cohort of patients with Gitelman syndrome.* Eur J Hum Genet, 2012. **20**(3): p. 263-70.
312. Gutierrez, M., et al., *[Gitelman syndrome associated with chondrocalcinosis: description of two cases].* Reumatismo, 2010. **62**(1): p. 60-4.

313. Simon, D.B., et al., *Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter.* Nat Genet, 1996. **12**(1): p. 24-30.
314. Lemmink, H.H., et al., *Linkage of Gitelman syndrome to the thiazide-sensitive sodium-chloride cotransporter gene with identification of mutations in Dutch families.* Pediatr Nephrol, 1996. **10**(4): p. 403-7.
315. Lemmink, H.H., et al., *Novel mutations in the thiazide-sensitive NaCl cotransporter gene in patients with Gitelman syndrome with predominant localization to the C-terminal domain.* Kidney Int, 1998. **54**(3): p. 720-30.
316. Vargas-Poussou, R., et al., *Spectrum of mutations in Gitelman syndrome.* J Am Soc Nephrol, 2011. **22**(4): p. 693-703.
317. Jeck, N., et al., *Mutations in the chloride channel gene, CLCNKB, leading to a mixed Bartter-Gitelman phenotype.* Pediatr Res, 2000. **48**(6): p. 754-8.
318. Enriquez, R., et al., *Gitelman syndrome due to p.A204T mutation in CLCNKB gene.* Int Urol Nephrol, 2010. **42**(4): p. 1099-102.
319. Knoers, N.V. and E.N. Levtchenko, *Gitelman syndrome.* Orphanet J Rare Dis, 2008. **3**: p. 22.
320. Hisakawa, N., et al., *A case of Gitelman's syndrome with chondrocalcinosis.* Endocr J, 1998. **45**(2): p. 261-7.
321. Punzi, L., et al., *Chondrocalcinosis is a feature of Gitelman's variant of Bartter's syndrome. A new look at the hypomagnesemia associated with calcium pyrophosphate dihydrate crystal deposition disease.* Rev Rhum Engl Ed, 1998. **65**(10): p. 571-4.
322. Cobeta-Garcia, J.C., et al., *Chondrocalcinosis and Gitelman's syndrome. A new association?* Ann Rheum Dis, 1998. **57**(12): p. 748-9.
323. Vezzoli, G., et al., *Choroidal calcifications in patients with Gitelman's syndrome.* Am J Kidney Dis, 2000. **36**(4): p. 855-8.
324. Garcia Nieto, V., et al., *[Chondrocalcinosis and hypomagnesaemia in a patient with a new mutation in the gene of the thiazide-sensitive Na-Cl cotransporter]*. Nefrologia, 2003. **23**(6): p. 504-9.
325. Ea, H.K., et al., *Chondrocalcinosis secondary to hypomagnesemia in Gitelman's syndrome.* J Rheumatol, 2005. **32**(9): p. 1840-2.
326. Gupta, R., et al., *Sclerochoroidal calcification associated with Gitelman syndrome and calcium pyrophosphate dihydrate deposition.* J Clin Pathol, 2005. **58**(12): p. 1334-5.
327. Hashida, T., et al., *Loss of consciousness and hypokalemia in an elderly man with a mutation of the thiazide-sensitive Na-Cl cotransporter gene.* Endocr J, 2006. **53**(6): p. 859-63.
328. Volpe, A., et al., *Familiar association of Gitelman's syndrome and calcium pyrophosphate dihydrate crystal deposition disease--a case report.* Rheumatology (Oxford), 2007. **46**(9): p. 1506-8.
329. Gutierrez, M., et al., *Gitelman syndrome, calcium pyrophosphate dihydrate deposition disease and crowned dens syndrome. A new association?* Rheumatology (Oxford), 2010. **49**(3): p. 610-3.
330. Rim, P.C. and M.P. Keith, *Chondrocalcinosis and hypomagnesemia in a 26-year-old woman.* J Clin Rheumatol, 2011. **17**(6): p. 334-5.
331. Iqbal, Z. and J.A. Sayer, *Chondrocalcinosis and Gitelman syndrome.* QJM, 2016.
332. Richette, P., et al., *Hypomagnesemia associated with chondrocalcinosis: a cross-sectional study.* Arthritis Rheum, 2007. **57**(8): p. 1496-501.

333. Favero, M., et al., *Miscellaneous non-inflammatory musculoskeletal conditions. Bartter's and Gitelman's diseases.* Best Pract Res Clin Rheumatol, 2011. **25**(5): p. 637-48.
334. Bettinelli, A., et al., *Genetic heterogeneity in tubular hypomagnesemia-hypokalemia with hypocalcuria (Gitelman's syndrome).* Kidney Int, 1995. **47**(2): p. 547-51.
335. Cruz, D.N., et al., *Gitelman's syndrome revisited: an evaluation of symptoms and health-related quality of life.* Kidney Int, 2001. **59**(2): p. 710-7.
336. Yagi, H., et al., *Inheritance of an autosomal recessive disorder, Gitelman's syndrome, across two generations in one family.* Intern Med, 2011. **50**(11): p. 1211-4.
337. Hahn, M., et al., *Chronic calcium pyrophosphate crystal inflammatory arthritis induced by extreme hypomagnesemia in short bowel syndrome.* BMC Gastroenterol, 2012. **12**: p. 129.
338. Nakhoul, F., et al., *Gitelman's syndrome: a pathophysiological and clinical update.* Endocrine, 2012. **41**(1): p. 53-7.
339. Syren, M.L., et al., *Identification of fifteen novel mutations in the SLC12A3 gene encoding the Na-Cl Co-transporter in Italian patients with Gitelman syndrome.* Hum Mutat, 2002. **20**(1): p. 78.
340. Mastroianni, N., et al., *Novel molecular variants of the Na-Cl cotransporter gene are responsible for Gitelman syndrome.* Am J Hum Genet, 1996. **59**(5): p. 1019-26.
341. Altman, R.D., et al., *Articular chondrocalcinosis. Microanalysis of pyrophosphate (PPi) in synovial fluid and plasma.* Arthritis Rheum, 1973. **16**(2): p. 171-8.
342. Silcox, D.C. and D.J. McCartyJr, *Elevated inorganic concentrations in synovial fluids in osteoarthritis and pseudogout.* J Lab Clin Med, 1974. **83**(4).
343. Williams, C.J., et al., *Autosomal dominant familial calcium pyrophosphate dihydrate deposition disease is caused by mutation in the transmembrane protein ANKH.* Am J Hum Genet, 2002. **71**(4): p. 985-91.
344. Williams, C.J., et al., *Mutations in the amino terminus of ANKH in two US families with calcium pyrophosphate dihydrate crystal deposition disease.* Arthritis Rheum, 2003. **48**(9): p. 2627-31.
345. Gruber, B.L., et al., *Novel ANKH amino terminus mutation (Pro5Ser) associated with early-onset calcium pyrophosphate disease with associated phosphaturia.* J Clin Rheumatol, 2012. **18**(4): p. 192-5.
346. Nurnberg, P., et al., *Heterozygous mutations in ANKH, the human ortholog of the mouse progressive ankylosis gene, result in craniometaphyseal dysplasia.* Nat Genet, 2001. **28**(1): p. 37-41.
347. Kornak, U., et al., *Three novel mutations in the ANK membrane protein cause craniometaphyseal dysplasia with variable conductive hearing loss.* Am J Med Genet A, 2010. **152A**(4): p. 870-4.
348. Dutra, E.H., et al., *Two novel large ANKH deletion mutations in sporadic cases with craniometaphyseal dysplasia.* Clin Genet, 2012. **81**(1): p. 93-5.
349. Nakajima, M., et al., *Identification and Functional Characterization of RSPO2 as a Susceptibility Gene for Ossification of the Posterior Longitudinal Ligament of the Spine.* Am J Hum Genet, 2016. **99**(1): p. 202-7.
350. Knight, M.N. and K.D. Hankenson, *R-spondins: novel matricellular regulators of the skeleton.* Matrix Biol, 2014. **37**: p. 157-61.
351. Bruchle, N.O., et al., *RSPO4 is the major gene in autosomal-recessive anonychia and mutations cluster in the furin-like cysteine-rich domains of the Wnt signaling ligand R-spondin 4.* J Invest Dermatol, 2008. **128**(4): p. 791-6.

352. Lin, F., et al., *MAN1, an integral protein of the inner nuclear membrane, binds Smad2 and Smad3 and antagonizes transforming growth factor-beta signaling.* Hum Mol Genet, 2005. **14**(3): p. 437-45.
353. Korman, B., et al., *Mutation in LEMD3 (Man1) Associated with Osteopoikilosis and Late-Onset Generalized Morphea: A New Buschke-Ollendorf Syndrome Variant.* Case Rep Dermatol Med, 2016. **2016**: p. 2483041.
354. Purcell, S., et al., *PLINK: a tool set for whole-genome association and population-based linkage analyses.* Am J Hum Genet, 2007. **81**(3): p. 559-75.
355. Blaydon, D.C., et al., *The gene encoding R-spondin 4 (RSPO4), a secreted protein implicated in Wnt signaling, is mutated in inherited anonychia.* Nat Genet, 2006. **38**(11): p. 1245-7.
356. van der Korst, J.K. and J. Geerards, *Articular chondrocalcinosis in a Dutch pedigree.* Arthritis Rheum, 1976. **19 Suppl 3**: p. 405-9.
357. Harris, J., et al., *Ankylosing hyperostosis. I. Clinical and radiological features.* Ann Rheum Dis, 1974. **33**(3): p. 210-5.
358. Kim, K.A., et al., *R-Spondin proteins: a novel link to beta-catenin activation.* Cell Cycle, 2006. **5**(1): p. 23-6.
359. Kim, K.A., et al., *R-Spondin family members regulate the Wnt pathway by a common mechanism.* Mol Biol Cell, 2008. **19**(6): p. 2588-96.
360. Senolt, L., et al., *Low circulating Dickkopf-1 and its link with severity of spinal involvement in diffuse idiopathic skeletal hyperostosis.* Ann Rheum Dis, 2012. **71**(1): p. 71-4.
361. Kim, J.H., et al., *Wnt signaling in bone formation and its therapeutic potential for bone diseases.* Ther Adv Musculoskelet Dis, 2013. **5**(1): p. 13-31.
362. Kielkopf, C.L., S. Lucke, and M.R. Green, *U2AF homology motifs: protein recognition in the RRM world.* Genes Dev, 2004. **18**(13): p. 1513-26.
363. Caputo, S., et al., *The carboxyl-terminal nucleoplasmic region of MAN1 exhibits a DNA binding winged helix domain.* J Biol Chem, 2006. **281**(26): p. 18208-15.
364. Konde, E., et al., *Structural analysis of the Smad2-MAN1 interaction that regulates transforming growth factor-beta signaling at the inner nuclear membrane.* Biochemistry, 2010. **49**(37): p. 8020-32.
365. Raju, G.P., et al., *SANE, a novel LEM domain protein, regulates bone morphogenetic protein signaling through interaction with Smad1.* J Biol Chem, 2003. **278**(1): p. 428-37.
366. Ng, S.B., et al., *Exome sequencing identifies the cause of a mendelian disorder.* Nat Genet, 2010. **42**(1): p. 30-5.
367. Rabbani, B., M. Tekin, and N. Mahdieh, *The promise of whole-exome sequencing in medical genetics.* J Hum Genet, 2014. **59**(1): p. 5-15.
368. Weinfeld, R.M., et al., *The prevalence of diffuse idiopathic skeletal hyperostosis (DISH) in two large American Midwest metropolitan hospital populations.* Skeletal Radiol, 1997. **26**(4): p. 222-5.
369. Cooper, G.M., et al., *Distribution and intensity of constraint in mammalian genomic sequence.* Genome Res, 2005. **15**(7): p. 901-13.
370. Mao, D., et al., *PLCgamma2 regulates osteoclastogenesis via its interaction with ITAM proteins and GAB2.* J Clin Invest, 2006. **116**(11): p. 2869-79.
371. Glaudo, M., et al., *Heterozygous inactivating CaSR mutations causing neonatal hyperparathyroidism: function, inheritance and phenotype.* Eur J Endocrinol, 2016. **175**(5): p. 421-31.
372. Boskey, A.L. and A.S. Posner, *Bone structure, composition, and mineralization.* Orthop Clin North Am, 1984. **15**(4): p. 597-612.

373. Vorgerd, M., et al., *A mutation in the dimerization domain of filamin c causes a novel type of autosomal dominant myofibrillar myopathy.* Am J Hum Genet, 2005. **77**(2): p. 297-304.
374. Duff, R.M., et al., *Mutations in the N-terminal actin-binding domain of filamin C cause a distal myopathy.* Am J Hum Genet, 2011. **88**(6): p. 729-40.
375. Bowles, N.E., et al., *Exome analysis of a family with Wolff-Parkinson-White syndrome identifies a novel disease locus.* Am J Med Genet A, 2015. **167A**(12): p. 2975-84.
376. Chen, D., M. Zhao, and G.R. Mundy, *Bone morphogenetic proteins.* Growth Factors, 2004. **22**(4): p. 233-41.
377. Ono, K., et al., *Pathology of ossification of the posterior longitudinal ligament and ligamentum flavum.* Clin Orthop Relat Res, 1999(359): p. 18-26.
378. Kan, L., et al., *Transgenic mice overexpressing BMP4 develop a fibrodysplasia ossificans progressiva (FOP)-like phenotype.* Am J Pathol, 2004. **165**(4): p. 1107-15.
379. Guo, X., et al., *Recombinant human bone morphogenetic protein-4 (rhBMP-4) enhanced posterior spinal fusion without decortication.* J Orthop Res, 2002. **20**(4): p. 740-6.
380. Suh, J.S., et al., *Coding polymorphisms of bone morphogenetic protein 2 contribute to the development of childhood IgA nephropathy.* Exp Ther Med, 2011. **2**(2): p. 337-341.
381. Capasso, M., et al., *A predicted functional single-nucleotide polymorphism of bone morphogenetic protein-4 gene affects mRNA expression and shows a significant association with cutaneous melanoma in Southern Italian population.* J Cancer Res Clin Oncol, 2009. **135**(12): p. 1799-807.
382. Tanno, M., et al., *Uniaxial cyclic stretch induces osteogenic differentiation and synthesis of bone morphogenetic proteins of spinal ligament cells derived from patients with ossification of the posterior longitudinal ligaments.* Bone, 2003. **33**(4): p. 475-84.
383. Ramesh Babu, L., et al., *Bone mass effects of a BMP4 gene polymorphism in postmenopausal women.* Bone, 2005. **36**(3): p. 555-61.
384. Montero, A., et al., *Disruption of the fibroblast growth factor-2 gene results in decreased bone mass and bone formation.* J Clin Invest, 2000. **105**(8): p. 1085-93.
385. Lei, S.F., C.J. Papasian, and H.W. Deng, *Polymorphisms in predicted miRNA binding sites and osteoporosis.* J Bone Miner Res, 2011. **26**(1): p. 72-8.
386. Wang, X.J., et al., *Prediction and identification of Arabidopsis thaliana microRNAs and their mRNA targets.* Genome Biol, 2004. **5**(9): p. R65.
387. Li, D., et al., *Associations between microRNA binding site SNPs in FGFs and FGFRs and the risk of non-syndromic orofacial cleft.* Sci Rep, 2016. **6**: p. 31054.
388. Giachelli, C.M., *Ectopic calcification: gathering hard facts about soft tissue mineralization.* Am J Pathol, 1999. **154**(3): p. 671-5.
389. Tarling, E.J., T.Q. de Aguiar Vallim, and P.A. Edwards, *Role of ABC transporters in lipid transport and human disease.* Trends Endocrinol Metab, 2013. **24**(7): p. 342-50.
390. Nitschke, Y., et al., *Generalized arterial calcification of infancy and pseudoxanthoma elasticum can be caused by mutations in either ENPP1 or ABCC6.* Am J Hum Genet, 2012. **90**(1): p. 25-39.
391. Hessle, L., et al., *Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization.* Proc Natl Acad Sci U S A, 2002. **99**(14): p. 9445-9.
392. Costrop, L.M., et al., *Novel deletions causing pseudoxanthoma elasticum underscore the genomic instability of the ABCC6 region.* J Hum Genet, 2010. **55**(2): p. 112-7.

393. Madon, J., et al., *Transport function and hepatocellular localization of mrp6 in rat liver.* Mol Pharmacol, 2000. **57**(3): p. 634-41.
394. Maher, J.M., et al., *Tissue distribution and hepatic and renal ontogeny of the multidrug resistance-associated protein (Mrp) family in mice.* Drug Metab Dispos, 2005. **33**(7): p. 947-55.
395. Beck, K., et al., *The distribution of Abcc6 in normal mouse tissues suggests multiple functions for this ABC transporter.* J Histochem Cytochem, 2003. **51**(7): p. 887-902.
396. Jiang, Q., et al., *Parabiotic heterogenetic pairing of Abcc6-/-/Rag1-/- mice and their wild-type counterparts halts ectopic mineralization in a murine model of pseudoxanthoma elasticum.* Am J Pathol, 2010. **176**(4): p. 1855-62.
397. Jansen, R.S., et al., *ABCC6-mediated ATP secretion by the liver is the main source of the mineralization inhibitor inorganic pyrophosphate in the systemic circulation-brief report.* Arterioscler Thromb Vasc Biol, 2014. **34**(9): p. 1985-9.
398. Lomashvili, K.A., et al., *Vascular calcification is dependent on plasma levels of pyrophosphate.* Kidney Int, 2014. **85**(6): p. 1351-6.
399. Dabisch-Ruthe, M., et al., *Pyrophosphates as a major inhibitor of matrix calcification in Pseudoxanthoma elasticum.* J Dermatol Sci, 2014. **75**(2): p. 109-20.
400. Terkeltaub, R.A., *Inorganic pyrophosphate generation and disposition in pathophysiology.* Am J Physiol Cell Physiol, 2001. **281**(1): p. C1-C11.
401. Henthorn, P.S., et al., *Different missense mutations at the tissue-nonspecific alkaline phosphatase gene locus in autosomal recessively inherited forms of mild and severe hypophosphatasia.* Proc Natl Acad Sci U S A, 1992. **89**(20): p. 9924-8.
402. Pulkkinen, L., et al., *Identification of ABCC6 pseudogenes on human chromosome 16p: implications for mutation detection in pseudoxanthoma elasticum.* Hum Genet, 2001. **109**(3): p. 356-65.
403. Lopa, S., et al., *Stability of housekeeping genes in human intervertebral disc, endplate and articular cartilage cells in multiple conditions for reliable transcriptional analysis.* Eur Cell Mater, 2016. **31**: p. 395-406.
404. Al-Sabah, A., et al., *Importance of reference gene selection for articular cartilage mechanobiology studies.* Osteoarthritis Cartilage, 2016. **24**(4): p. 719-30.
405. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
406. Schulz, V., et al., *Mutational analysis of the ABCC6 gene and the proximal ABCC6 gene promoter in German patients with pseudoxanthoma elasticum (PXE).* Hum Mutat, 2006. **27**(8): p. 831.
407. Le Saux, O., et al., *A spectrum of ABCC6 mutations is responsible for pseudoxanthoma elasticum.* Am J Hum Genet, 2001. **69**(4): p. 749-64.
408. Gran, J.T., et al., *Radiological changes in men and women with ankylosing spondylitis.* Ann Rheum Dis, 1984. **43**(4): p. 570-5.
409. Ciurea, A., et al., *Age at symptom onset in ankylosing spondylitis: is there a gender difference?* Ann Rheum Dis, 2014. **73**(10): p. 1908-10.
410. van der Horst-Bruinsma, I.E., et al., *Female patients with ankylosing spondylitis: analysis of the impact of gender across treatment studies.* Ann Rheum Dis, 2013. **72**(7): p. 1221-4.
411. Hoyle, E., et al., *The X-chromosome and susceptibility to ankylosing spondylitis.* Arthritis Rheum, 2000. **43**(6): p. 1353-5.
412. Higgins, C.F., *ABC transporters: physiology, structure and mechanism--an overview.* Res Microbiol, 2001. **152**(3-4): p. 205-10.

413. Wang, J., et al., *ABCC6 gene polymorphism associated with variation in plasma lipoproteins.* J Hum Genet, 2001. **46**(12): p. 699-705.
414. Peloso, G.M., et al., *Common genetic variation in multiple metabolic pathways influences susceptibility to low HDL-cholesterol and coronary heart disease.* J Lipid Res, 2010. **51**(12): p. 3524-32.
415. Gorgels, T.G., et al., *Disruption of Abcc6 in the mouse: novel insight in the pathogenesis of pseudoxanthoma elasticum.* Hum Mol Genet, 2005. **14**(13): p. 1763-73.
416. Pohl, A., P.F. Devaux, and A. Herrmann, *Function of prokaryotic and eukaryotic ABC proteins in lipid transport.* Biochim Biophys Acta, 2005. **1733**(1): p. 29-52.
417. Germain, D.P., et al., *Homozygosity for the R1268Q mutation in MRP6, the pseudoxanthoma elasticum gene, is not disease-causing.* Biochem Biophys Res Commun, 2000. **274**(2): p. 297-301.
418. Dequeker, J. and P. Dieppe, *Disorders of Bone, Cartilage and Connective Tissue*, in *Rheumatology*, J.H. Klippel and P.A. Dieppe, Editors. 1998, Mosby: London.
419. Yoshihara, H., et al., *Rheumatoid arthritis patients undergoing total hip and knee arthroplasty have better in-hospital outcomes compared with non-rheumatoid arthritis patients.* Clin Exp Rheumatol, 2016. **34**(2): p. 270-5.
420. Rotes-Querol, J., *Clinical manifestations of diffuse idiopathic skeletal hyperostosis (DISH).* Br J Rheumatol, 1996. **35**(12): p. 1193-4.
421. Musacchio, E., et al., *The impact of knee and hip chondrocalcinosis on disability in older people: the ProVA Study from northeastern Italy.* Ann Rheum Dis, 2011. **70**(11): p. 1937-43.
422. Crawford, R., et al., *Deposition of calcium pyrophosphate in tissue after revision arthroplasty of the hip.* J Bone Joint Surg Br, 1999. **81**(3): p. 552-4.
423. Blazquez-Medela, A.M., et al., *ABCC6 deficiency is associated with activation of BMP signaling in liver and kidney.* FEBS Open Bio, 2015. **5**: p. 257-63.
424. Hosen, M.J., et al., *Perturbation of specific pro-mineralizing signalling pathways in human and murine pseudoxanthoma elasticum.* Orphanet J Rare Dis, 2014. **9**: p. 66.
425. Kidd, P.M., *Vitamins D and K as pleiotropic nutrients: clinical importance to the skeletal and cardiovascular systems and preliminary evidence for synergy.* Altern Med Rev, 2010. **15**(3): p. 199-222.
426. Zhai, Y., et al., *Expression and function of matrix Gla protein in human peritoneal mesothelial cells.* Nephrol Dial Transplant, 2010. **25**(10): p. 3213-21.
427. Li, Q., et al., *Pseudoxanthoma elasticum: reduced gamma-glutamyl carboxylation of matrix gla protein in a mouse model (Abcc6-/-).* Biochem Biophys Res Commun, 2007. **364**(2): p. 208-13.
428. Gheduzzi, D., et al., *Matrix Gla protein is involved in elastic fiber calcification in the dermis of pseudoxanthoma elasticum patients.* Lab Invest, 2007. **87**(10): p. 998-1008.
429. Hendig, D., et al., *The local calcification inhibitor matrix Gla protein in pseudoxanthoma elasticum.* Clin Biochem, 2008. **41**(6): p. 407-12.
430. Rutsch, F. and R. Terkeltaub, *Parallels between arterial and cartilage calcification: what understanding artery calcification can teach us about chondrocalcinosis.* Curr Opin Rheumatol, 2003. **15**(3): p. 302-10.
431. Zebboudj, A.F., M. Imura, and K. Bostrom, *Matrix GLA protein, a regulatory protein for bone morphogenetic protein-2.* J Biol Chem, 2002. **277**(6): p. 4388-94.
432. Sarzi-Puttini, P., et al., *Matrix GLA protein: evidence of persistently increased concentrations in DISH patients.* Arthritis Rheum, 2003(48): p. S534.

433. Li, X., H.Y. Yang, and C.M. Giachelli, *BMP-2 promotes phosphate uptake, phenotypic modulation, and calcification of human vascular smooth muscle cells.* Atherosclerosis, 2008. **199**(2): p. 271-7.
434. Tada, H., et al., *Phosphate increases bone morphogenetic protein-2 expression through cAMP-dependent protein kinase and ERK1/2 pathways in human dental pulp cells.* Bone, 2011. **48**(6): p. 1409-16.
435. Vasiliou, V., K. Vasiliou, and D.W. Nebert, *Human ATP-binding cassette (ABC) transporter family.* Hum Genomics, 2009. **3**(3): p. 281-90.
436. Vanakker, O.M., M.J. Hosen, and A.D. Paepe, *The ABCC6 transporter: what lessons can be learnt from other ATP-binding cassette transporters?* Front Genet, 2013. **4**: p. 203.
437. Higgins, C.F., *ABC transporters: from microorganisms to man.* Annu Rev Cell Biol, 1992. **8**: p. 67-113.
438. Hung, L.W., et al., *Crystal structure of the ATP-binding subunit of an ABC transporter.* Nature, 1998. **396**(6712): p. 703-7.
439. Hollenstein, K., R.J. Dawson, and K.P. Locher, *Structure and mechanism of ABC transporter proteins.* Curr Opin Struct Biol, 2007. **17**(4): p. 412-8.
440. Verrier, P.J., et al., *Plant ABC proteins--a unified nomenclature and updated inventory.* Trends Plant Sci, 2008. **13**(4): p. 151-9.
441. Stefkova, J., R. Poledne, and J.A. Hubacek, *ATP-binding cassette (ABC) transporters in human metabolism and diseases.* Physiol Res, 2004. **53**(3): p. 235-43.
442. Simith, R. and P. Wordsworth, *The physiology and pathology of bone*, in *Clinical and Biochemical Disorders of the Skeleton*. 2005, Oxford University Press: Oxford.
443. Dean, M., Y. Hamon, and G. Chimini, *The human ATP-binding cassette (ABC) transporter superfamily.* J Lipid Res, 2001. **42**(7): p. 1007-17.
444. Costa, R.A., J.C. Cardoso, and D.M. Power, *Evolution of the angiopoietin-like gene family in teleosts and their role in skin regeneration.* BMC Evol Biol, 2017. **17**(1): p. 14.
445. Fujita, P.A., et al., *The UCSC Genome Browser database: update 2011.* Nucleic Acids Res, 2011. **39**(Database issue): p. D876-82.
446. Abascal, F., R. Zardoya, and D. Posada, *ProtTest: selection of best-fit models of protein evolution.* Bioinformatics, 2005. **21**(9): p. 2104-5.
447. Guindon, S., et al., *New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0.* Syst Biol, 2010. **59**(3): p. 307-21.
448. Louro, B., et al., *Having a BLAST: Searchable transcriptome resources for the gilthead sea bream and the European sea bass.* Mar Genomics, 2016. **30**: p. 67-71.
449. Levasseur, A. and P. Pontarotti, *The role of duplications in the evolution of genomes highlights the need for evolutionary-based approaches in comparative genomics.* Biol Direct, 2011. **6**: p. 11.
450. Caputo Barucchi, V., et al., *Genome duplication in early vertebrates: insights from agnathan cytogenetics.* Cytogenet Genome Res, 2013. **141**(2-3): p. 80-9.
451. Volff, J.N., *Genome evolution and biodiversity in teleost fish.* Heredity (Edinb), 2005. **94**(3): p. 280-94.
452. Sato, Y., Y. Hashiguchi, and M. Nishida, *Temporal pattern of loss/persistence of duplicate genes involved in signal transduction and metabolic pathways after teleost-specific genome duplication.* BMC Evol Biol, 2009. **9**: p. 127.
453. Qian, W., et al., *Maintenance of duplicate genes and their functional redundancy by reduced expression.* Trends Genet, 2010. **26**(10): p. 425-30.

454. Hosen, M.J., et al., *Zebrafish models for ectopic mineralization disorders: practical issues from morpholino design to post-injection observations.* Front Genet, 2013. **4**: p. 74.
455. Geourjon, C., et al., *A common mechanism for ATP hydrolysis in ABC transporter and helicase superfamilies.* Trends Biochem Sci, 2001. **26**(9): p. 539-44.
456. Slatkin, M., *A population-genetic test of founder effects and implications for Ashkenazi Jewish diseases.* Am J Hum Genet, 2004. **75**(2): p. 282-93.
457. Thompson, E.A. and J.V. Neel, *Private polymorphisms: how many? How old? How useful for genetic taxonomies?* Mol Phylogenet Evol, 1996. **5**(1): p. 220-31.
458. Raza, S., et al., *GLY67ARG substitution in RSPO4 disrupts the WNT signaling pathway due to an abnormal binding pattern with LGRs leading to anonychia.* RSC Adv, 2017. **7**(28): p. 17357-17366.
459. Olson, M., *Enrichment of super-sized resequencing targets from the human genome.* Nat Methods, 2007. **4**(11): p. 891-2.
460. Shore, E.M., et al., *A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva.* Nat Genet, 2006. **38**(5): p. 525-7.
461. Sun, Y., et al., *A recurrent mutation c.617G>A in the ACVR1 gene causes fibrodysplasia ossificans progressiva in two Chinese patients.* Calcif Tissue Int, 2009. **84**(5): p. 361-5.
462. Botstein, D. and N. Risch, *Discovering genotypes underlying human phenotypes: past successes for mendelian disease, future approaches for complex disease.* Nat Genet, 2003. **33 Suppl**: p. 228-37.
463. Chen, G., C. Deng, and Y.P. Li, *TGF-beta and BMP signaling in osteoblast differentiation and bone formation.* Int J Biol Sci, 2012. **8**(2): p. 272-88.
464. Hu, Y.Y., et al., *Association between BMP4 rs17563 polymorphism and NSCL/P risk: a meta-analysis.* Dis Markers, 2015. **2015**: p. 763090.
465. Savitha, S., et al., *Single nucleotide polymorphism of bone morphogenetic protein 4 gene: A risk factor of non-syndromic cleft lip with or without palate.* Indian J Plast Surg, 2015. **48**(2): p. 159-64.
466. Lin, J.Y., et al., *Association of bone morphogenetic protein 4 gene polymorphisms with nonsyndromic cleft lip with or without cleft palate in Chinese children.* DNA Cell Biol, 2008. **27**(11): p. 601-5.
467. Araujo, T.K., et al., *Preliminary Analysis of the Nonsynonymous Polymorphism rs17563 in BMP4 Gene in Brazilian Population Suggests Protection for Nonsyndromic Cleft Lip and Palate.* Plast Surg Int, 2012. **2012**: p. 247104.
468. Murthy, J. and L. Bhaskar, *Current concepts in genetics of nonsyndromic clefts.* Indian J Plast Surg, 2009. **42**(1): p. 68-81.
469. Prescott, E.T. and I.N. Mungrue, *ABCC6 Out From the Cold: Identification of the ABCC6 Substrate as a Therapy for Pseudoxanthoma Elasticum and Cardiovascular Disease.* Austin J Pharmacol Ther, 2014. **2**(5).
470. Pomozi, V., et al., *ABCC6 is a basolateral plasma membrane protein.* Circ Res, 2013. **112**(11): p. e148-51.
471. Matsuzaki, Y., et al., *Tissue-specific expression of the ABCC6 gene.* J Invest Dermatol, 2005. **125**(5): p. 900-5.
472. Kool, M., et al., *Expression of human MRP6, a homologue of the multidrug resistance protein gene MRP1, in tissues and cancer cells.* Cancer Res, 1999. **59**(1): p. 175-82.
473. Thouverey, C., et al., *Inorganic pyrophosphate as a regulator of hydroxyapatite or calcium pyrophosphate dihydrate mineral deposition by matrix vesicles.* Osteoarthritis Cartilage, 2009. **17**(1): p. 64-72.

474. Smith, R. and P. Wordsworth, *Clinical and Biochemical disorders of the skeleton.* 1st ed ed. 2005, Oxford: Oxford University Press. 471.
475. Barskova, V.G., et al., *Comparison of three imaging techniques in diagnosis of chondrocalcinosis of the knees in calcium pyrophosphate deposition disease.* Rheumatology (Oxford), 2013. **52**(6): p. 1090-4.
476. Sauna, Z.E. and C. Kimchi-Sarfaty, *Understanding the contribution of synonymous mutations to human disease.* Nat Rev Genet, 2011. **12**(10): p. 683-91.
477. Strachan, T. and A. Read, *Human Molecular Genetics*. 4th ed. 2011, New York: Garland Science/Taylor & Francis Group.
478. Faa, V., et al., *A synonymous mutation in the CFTR gene causes aberrant splicing in an italian patient affected by a mild form of cystic fibrosis.* J Mol Diagn, 2010. **12**(3): p. 380-3.
479. Scott, A., et al., *Functional analysis of synonymous substitutions predicted to affect splicing of the CFTR gene.* J Cyst Fibros, 2012. **11**(6): p. 511-7.
480. Ward, L.D. and M. Kellis, *Interpreting noncoding genetic variation in complex traits and human disease.* Nat Biotechnol, 2012. **30**(11): p. 1095-106.
481. Duan, J., et al., *Synonymous mutations in the human dopamine receptor D2 (DRD2) affect mRNA stability and synthesis of the receptor.* Hum Mol Genet, 2003. **12**(3): p. 205-16.
482. Law, A.J., et al., *Disease-associated intronic variants in the ErbB4 gene are related to altered ErbB4 splice-variant expression in the brain in schizophrenia*. Hum Mol Genet, 2007. **16**(2): p. 129-41.

APPENDIX

Rheumatology: Current Research

Parreira et al., Rheumatology (Sunnyvale) 2015, 5:3
http://dx.doi.org/10.4172/2161-1149.1000163

Case Report Open Access

Gitelman's Syndrome Associated with Chondrocalcinosis: A Case Study from the Azores Islands (Portugal)

Parreira B[1,2,3*], **Couto AR**[1,2,3], **Menezes S**[3], **Machado V**[4], **Soares M**[1,2,3], **Pinheiro JP**[1,2,3], **Lima M**[1,2,5] **and Bruges-Armas J**[1,2,3]

[1]*University of Porto, Institute for Research and Innovation in Health Porto, Portugal*

[2]*University of Porto, IBMC - Institute for Molecular and Cell Biology, GARG- Genetics and Arthritis Research Group Porto, Portugal*

[3]*Santo Espírito Hospital of Terceira Island, EPE, Epidemiology and Molecular Biology Service (SEEBMO) Angra do Heroísmo, Portugal*

[4]*Santo Espírito Hospital of Terceira Island, EPE, Hospital Oncology service Angra do Heroísmo, Azores, Portugal*

[5]*University of Azores, Department of Biology, Ponta Delgada, Azores, Portugal*

[*]**Corresponding author:** Bruna Conceição Parreira, Researcher, Hospital de Santo Espirito da Ilha Terceira, Serviço Especializado de Epidemiologia e Biologia Molecular, Canada do Breado, 9700, Portugal, Tel: 00351968322116; Fax: 00351295216263; E-mail: bruna.parreira@seebmo.org

Received date: July 10, 2015; **Accepted date:** August 18, 2015; **Published date:** August 25, 2015

Abstract

Gitelman syndrome (GS) is a rare autossomal recessive inherited tubulopathy, characterized by defective tubular reabsorption of magnesium and potassium, mostly caused by mutations in the *SLC12A3* gene. The association of GS with chondrocalcinosis (CC) has been described in the literature as a typical example of hypomagnesemia-induced crystal deposition disease.

We are presenting one case where the genetic cause for GS was identified in a proband with secondary early onset CC. A 60 years-old male patient with CC, hypomagnesemia and hypokalemia was identified in our hospital as a result of clinical and laboratory assessments. The clinical diagnosis of GS was performed and *SLC12A3* gene was screened in the proband; variants detected were further searched in family members.

The proband was homozygous for the S615L mutation; additionally, only one from the seven family members which were heterozygous presents CC. The presence of CC in two other individuals is most likely sporadic, in agreement with their advanced age.

Keywords: Chondrocalcinosis; Calcium pyrophosphate dehydrate; Genetic studies

Introduction

Gitelman Syndrome (GS, OMIM 263800; ORPHA358) is a rare autossomal recessive tubulopathy, with a prevalence of approximately 1:40 000 in the Caucasian population. Onset is usually in adult life, but cases with onset in childhood are also known [1]. GS is characterized by hypomagnesemia, hypokalemia, metabolic alkalosis, hypocalciuria and hyperreninemic hyperaldosteronism. The clinical spectrum is wide and includes: cramps, myalgies, muscle weakness, tetania, and paralysis [2]. GS is mostly caused by loss of function mutations in the solute carrier family 12, member 3 (*SLC12A3* gene) which consists of 26 exons and is located on the long arm of 16th chromosome [1]; this gene encodes the thiazide-sensitive sodium-chloride cotransporter (NCCT), expressed in the distal convoluted tubule of the kidney [3].

NCCT is a polypeptide which consists of 1021 amino acids. Its 2-D structure is predicted to contain 12 transmembrane domains and intracellular amino and carboxyterminal regions [3]. At present, according to the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/), more than 100 different variants have been identified in patients with GS. They are missense, non-sense, frameshift, and splice-site variations and are scattered throughout the transporter protein with a possible clustering of variations in the carboxy-terminal tail [4].

The association of GS with chondrocalcinosis (CC) has been described in the literature as a typical example of hypomagnesemia-induced crystal deposition disease [2]. CC is characterized by deposition of crystals of calcium pyrophosphate dihydrate (CPPD) in articular hyaline and fibro-cartilage [2]. Seventeen cases of GS associated with CC due to CCPD have been published in the literature, including 10 females and 7 males with a mean age of 51.4 ± 15.9 years [2,5-15]. The role of hypomagnesemia in the development of CCPD is, however, not fully understood [16]. Magnesium is an important cofactor for alkaline pyrophosphatase, an enzyme that plays a key role by converting inorganic pyrophosphate (PPi) to orthophosphate (Pi). A reduction in the activity of this enzyme due to hypomagnesemia could induce CCPD by raising extracellular levels of PPi. Both PPi and calcium are crucial precursors for crystal nucleation. CCPD may be found in other conditions associated with hypomagnesemia, such as short bowel syndrome or in liver transplantation patients [16].

Case with Genetic Analysis

The proband, a 60 year-old caucasian male was first observed in the Rheumatic Diseases Clinic - HSEIT when he was 48 years old; he was born in Terceira island as well as both his parents. Symptoms started when he was 33 years old, mainly affecting knees, ankles, wrists, elbows and achilles tendons. In the proband, PPi crystals were identified in the synovial fluid aspirated from a right knee effusion. Since then he was under treatment with colchicine, NSAIDS (Nonsteroidal anti-inflammatory drugs), and oral potassium and

magnesium. Laboratory tests revealed normal leukocyte, erythrocyte and platelet count.

Blood urea was 33 mg/dl, creatinine 0.9 mg/dl and glucose 177 mg/dl. Serum electrolyte concentrations were as follows: sodium 139 mEq/L, potassium 3.2 mEq/L, calcium 9.8 mg/dl, and magnesium 1.1 mg/dl (Table 1). In spite of the treatment with colchicine, patient still maintain hypokalemia and hypomagnesemia, however he showed some improvements. Using the diagnostic criteria of Bettinelli et al. [17] a clinical diagnosis of GS was suspected, and a diagnosis of knee CC was made after the identification of bilateral knee cartilage calcification (Figure 1). SLC12A3 sequence analysis in the proband revealed that he was homozygous for a missense substitution in exon 15, previously described as associated to GS [18]. This mutation (CM014403), a C to T transition at position c.1869, changes the small size and polar amino acid serine to a medium size and hydrophobic leucine at position 615 (S615L), has a SIFT score of 0 and a Polyphen value of 0.996, both values suggestive of a deleterious variation.

Individuals	Sex	Age	Magnesium levels[a]	Potassium levels[b]	CC	Pathogenic mutation S615L
III.2	**M**	**60**	**1.1**	**3.2**	**+**	**S615L/S615L**
III.12	M	75	2.1	4.7	-	S615L/WT
III.13	F	67	2.2	4.1	-	WT/WT
III.16	M	75	2.0	4.6	+	WT/WT
III.19	F	79	1.9	4.7	+	S615L/WT
IV.1	M	35	2.3	3.7	-	S615L/WT
IV.2	F	54	2.2	4.2	-	S615L/WT
IV.4	M	46	1.9	4.0	-	S615L/WT
IV.14	F	51	1.9	4.0	-	WT/WT
IV.16	F	49	2.2	4.1	-	WT/WT
IV.17	F	45	1.9	4.3	-	WT/WT
IV.21	M	37	2.1	4.1	-	S615L/WT
IV.22	F	36	2.0	3.7	-	S615L/WT
V.1	F	25	2.0	4.7	-	WT/WT

[a]Normal serum magnesium 1.5-2.5 mg/dL; [b]Normal serum potassium; 3.3-5.1 mmol/L; M:Male; F:Female; CC:Chondrocalcinosis; WT:Wild Type

Table 1: Characteristics and *SLC12A3* gene variants identified in the proband and in thirteen individuals of his family pedigree. The proband is indicated by bold.

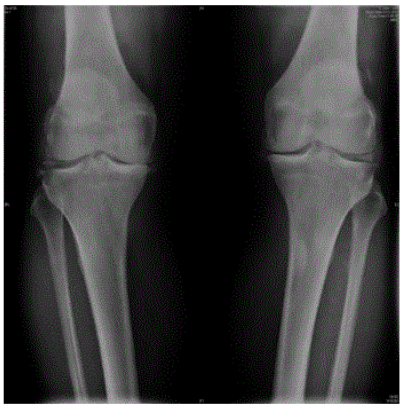


Figure 1: X-rays of proband showing classical chondrocalcinosis in knees.

Thirteen additional family members were investigated (5M: 8W; [25-79]; mean age 51); blood tests and x-rays were obtained for all of them (data not shown). The pedigree with investigated individuals is shown in Figure 2. The biochemical data in these patients show normal levels of serum magnesium ranging from 1.9 to 2.3 mg/dL and normal levels of potassium ranging from 3.7-4.7 mmol/L (Table 1).

When the S615L mutation was searched in the thirteen relatives of the proband, seven family members were found to be heterozygous, of which only one presented CC. Furthermore six individuals were wild-type homozygous; noteworthy, one of them (III.16) presented CC (Table 1 and Figure 2).

Discussion

The GS patient described in this study has the S615L variation in homozygosity, while the other cases of GS with this variation were reported in compound heterozygotes [2]. In our study, seven individuals heterozygous for the S615L did not have either hypokalemia or hypomagnesemia, confirming that they were asymptomatic carriers of this variation.

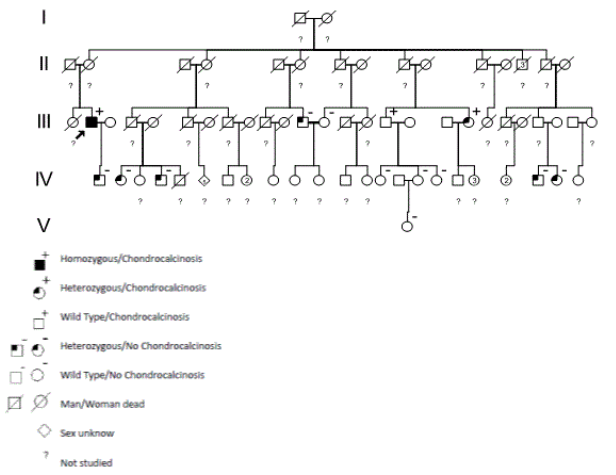


Figure 2: Pedigree with 14 investigated individuals (6 males and 8 females). The CC affected individuals and the presence of the mutation S615L are also indicated. The proband with GS is denoted by the arrow.

Hypomagnesemia and hypocalciuria are found in most cases of GS, however, some cases with mutations in the NCCT do not show these conditions [19]. It is believed that hypomagnesemia causes CC by increasing formation and reducing solubility of CCP crystals [16]. Excess of PPi is the main precursor for CPPD crystal nucleation. Because the magnesium is a necessary cofactor for numerous enzymes, such as pyrophosphatases, and considering the fact that it increases the solubility of CPPD crystals, low levels of magnesium could induce CPPD deposition disease by raising PPi and/or reducing the saturation product of CPPD crystals [14]. The prevalence of CC increases with age (10-15% for people between 65 and 75 years) and is hence called sporadic in patients older than 60 years, whereas in younger individuals there are several putative underlying disorders causing CPPD deposition disease, such as hemochromatosis, hyperparathyroidism, hypomagnesemia or hypophosphatemia [20].

The assumption that GS is caused by a defect in the NCCT cotransporter in the renal distal tubule has been proven by the identification of numerous variations in the *SLC12A3* gene in patients with GS [1,4,19,21]. In the present study the specific involvement of this cotransporter in the etiology of this disorder is further substantiated by the finding that the proband is homozygous for the S615L variation. The S615L identified in this study was previously described by Cruz and co-workers [18] in a study involved 36 kindreds from the United States, Canada and England and later reported in a study by Syrén et al. [22]. Although the *SLC12A3* variations reported in previous studies are distributed throughout the whole protein [4,23], the study of Lemmink (1998) indicates that the carboxy-terminal end represents a hot spot for variations [4]. S615L is located at the intracellular C-terminal end of the SLC12A3 protein. It is conceivable that structural alterations due to *SLC12A3* variations in the C-terminal domain interfere with phosphorylation of the NCCT protein and as such with its regulation [4].

Evidence for an association between CC and GS comes from uncontrolled case reports, case series and only one cross-sectional study. As a result, its epidemiology remains unknown 16. There have been few cases described with a definite diagnosis of CC due to GS. In some patients with CPPD deposition disease secondary to hypomagnesemia, the stabilization of magnesium and potassium levels can reduce the deposition of CPPD crystals in the synovium and synovial fluid, reducing the frequency of attacks of articular pain 14.

Conclusion

GS is a hereditary disease characterized by defective tubular reabsorption of magnesium and potassium, mostly caused by mutations in the *SLC12A3* gene. Sometimes GS patients, as in our case, might come with a CC diagnosis. We identified the genetic cause for GS in a proband with secondary early onset CC. Further studies are needed in order to enlighten the pathophysiology and prevalence of CC in patients with GS.

Acknowledgement

This study was partially supported by a PhD fellowship M3.1.2/F/023/2011 (Bruna Parreira) from Fundo Regional para a Ciência (FRC).

References

1. Glaudemans B, Yntema HG, San-Cristobal P, Schoots J, Pfundt R, et al. (2012) Novel NCC mutants and functional analysis in a new cohort of patients with Gitelman syndrome. Eur J Hum Genet 20: 263-270.
2. Gutierrez M, Silveri F, Bertolazzi C, Giacchetti G, Tardella M, et al. (2010) [Gitelman syndrome associated with chondrocalcinosis: description of two cases]. Reumatismo 62: 60-64.
3. Simon DB, Nelson-Williams C, Bia MJ, Ellison D, Karet FE, et al. (1996) Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. Nat Genet 12: 24-30.
4. Lemmink HH, Knoers NV, Károlyi L, van Dijk H, Niaudet P, et al. (1998) Novel mutations in the thiazide-sensitive NaCl cotransporter gene in patients with Gitelman syndrome with predominant localization to the C-terminal domain. Kidney Int 54: 720-730.
5. Hisakawa N, Yasuoka N, Itoh H, Takao T, Jinnouchi C, et al. (1998) A case of Gitelman's syndrome with chondrocalcinosis. Endocr J 45: 261-267.
6. Punzi L, Calò L, Schiavon F, Pianon M, Rosada M, et al. (1998) Chondrocalcinosis is a feature of Gitelman's variant of Bartter's syndrome. A new look at the hypomagnesemia associated with calcium pyrophosphate dihydrate crystal deposition disease. Rev Rhum Engl Ed 65: 571-574.
7. Cobeta-Garcia JC, Gascón A, Iglesias E, Estopiñán V (1998) Chondrocalcinosis and Gitelman's syndrome. A new association? Ann Rheum Dis 57: 748-749.
8. Vezzoli G, Soldati L, Jansen A, Pierro L (2000) Choroidal calcifications in patients with Gitelman's syndrome. Am J Kidney Dis 36: 855-858.
9. García Nieto V, Cantabrana A, Müller D, Claverie-Martín F (2003) [Chondrocalcinosis and hypomagnesaemia in a patient with a new mutation in the gene of the thiazide-sensitive Na-Cl cotransporter]. Nefrologia 23: 504-509.
10. Ea HK, Blanchard A, Dougados M, Roux C (2005) Chondrocalcinosis secondary to hypomagnesemia in Gitelman's syndrome. J Rheumatol 32: 1840-1842.
11. Gupta R, Hu V, Reynolds T, Harrison R (2005) Sclerochoroidal calcification associated with Gitelman syndrome and calcium pyrophosphate dihydrate deposition. J Clin Pathol 58: 1334-1335.
12. Hashida T, Yamada M, Hashimoto K, Satoh T, Okada S, et al. (2006) Loss of consciousness and hypokalemia in an elderly man with a mutation of the thiazide-sensitive Na-Cl cotransporter gene. Endocr J 53: 859-863.

13. Volpe A, Caramaschi P, Thalheimer U, Fava C, Ravagnani V, et al. (2007) Familiar association of Gitelman's syndrome and calcium pyrophosphate dihydrate crystal deposition disease - a case report. Rheumatology (Oxford) 46: 1506-1508.
14. Gutierrez M, Silveri F, Bertolazzi C, Salaffi F, Giacchetti G, et al. (2010) Gitelman syndrome, calcium pyrophosphate dihydrate deposition disease and crowned dens syndrome. A new association? Rheumatology (Oxford) 49: 610-613.
15. Koçkara AŞ, Candan F, Hüzmeli C, Kayataş M, Alaygut D (2013) Gitelman's syndrome associated with chondrocalcinosis: a case report. Ren Fail 35: 1285-1288.
16. Favero M, Calò LA, Schiavon F, Punzi L (2011) Miscellaneous non-inflammatory musculoskeletal conditions. Bartter's and Gitelman's diseases. Best Pract Res Clin Rheumatol 25: 637-648.
17. Bettinelli A, Bianchetti MG, Borella P, Volpini E, Metta MG, et al. (1995) Genetic heterogeneity in tubular hypomagnesemia-hypokalemia with hypocalcuria (Gitelman's syndrome). Kidney Int 47: 547-551.
18. Cruz DN, Shaer AJ, Bia MJ, Lifton RP, Simon DB; Yale Gitelman's and Bartter's Syndrome Collaborative Study Group (2001) Gitelman's syndrome revisited: an evaluation of symptoms and health-related quality of life. Kidney Int 59: 710-717.
19. Yagi H, Yahata K, Usui T, Hasegawa C, Seta K, et al. (2011) Inheritance of an autosomal recessive disorder, Gitelman's syndrome, across two generations in one family. Intern Med 50: 1211-1214.
20. Hahn M, Raithel M, Hagel A, Biermann T, Manger B (2012) Chronic calcium pyrophosphate crystal inflammatory arthritis induced by extreme hypomagnesemia in short bowel syndrome. BMC Gastroenterol 12: 129.
21. Nakhoul F, Nakhoul N, Dorman E, Berger L, Skorecki K, et al. (2012) Gitelman's syndrome: a pathophysiological and clinical update. Endocrine 41: 53-57.
22. Syrén ML, Tedeschi S, Cesareo L, Bellantuono R, Colussi G, et al. (2002) Identification of fifteen novel mutations in the SLC12A3 gene encoding the Na-Cl Co-transporter in Italian patients with Gitelman syndrome. Hum Mutat 20: 78.
23. Mastroianni N, Bettinelli A, Bianchetti M, Colussi G, De Fusco M, et al. (1996) Novel molecular variants of the Na-Cl cotransporter gene are responsible for Gitelman syndrome. Am J Hum Genet 59: 1019-1026.