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GENETICS AND EPIDEMIOLOGY OF ECTOPIC CALCIFICATION

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De acordo com o disposto no nº2 do Artigo 8º do Decreto-Lei nº388/70, nesta dissertação foram utilizados os resultados dos trabalhos publicados ou em vias de publicação abaixo indicados. No cumprimento do disposto no referido Decreto-Lei, a autora desta dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação dos resultados e redacção dos resultados publicados ou em preparação, sob o nome de Couto AR:

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LIST OF ABBREVIATIONS

ACVR1 – activin A receptor type I	GAG – glycosaminoglycan
ADHR - autosomal dominant hypophosphatemic rickets	GALNT3 – GalNAc transferase 3
AHO – Albright hereditary osteodystrophy	GNAS1 – guanine nucleotide-binding protein G(s) subunit alpha isoforms XLas
ALPL – alkaline phosphatase liver	GWAS – genome wide association study
AS – ankylosing spondylitis	HA – hydroxyapatite
ASARM - acidic serine- and aspartate-rich MEPE-associated motif	HAMP – hepcidin precursor
ATP7B – copper-transporting ATPase 2	HFE – hereditary haemochromatosis protein precursor
BCP – basic calcium phosphate	HGD – homogentisate 1,2-dioxygenase
BMPs – bone morphogenetic protein	HJV – hemojuvelin precursor
BOS – Buschke-Ollendorf syndrome	HLA – human leukocyte antigen
CASR – calcium sensing receptor	HRPT2 – hyperparathyroidism 2
CC – chondrocalcinosis	HRPT3 – hyperparathyroidism 3
CILP/NTPPH – cartilage intermediated layer protein/nucleotide pyrophosphohydrolase	IBD – identical by descent
CLCNKB – chloride channel protein CIC-Kb	IBS – identical by state
COL11A2 – collagen type XI, alpha 2	IGF – insulin like growth factor
COL2A1 – collagen type II, alpha 1	IL23 – interleukin 23
COL6A1 – collagen type VI, alpha 1	LEMD3 – LEM domain containing 3
COMP – cartilage oligomeric matrix protein	LIMK1 – LIM domain kinase 1
CPDD – calcium pyrophosphate deposition disease	LMNA – lamin A/C
CPPD – calcium pyrophosphate dehydrate	LMSv2 – linkage mapping set version 2
CREST – calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactily, telangiectasias	MED – multiple epiphyseal dysplasia
CS – chondroitin sulphate	MEN1 – multiple endocrine neoplasia 1
DISH – diffuse idiopathic skeletal hyperostosis	MEPE – matrix extracellular phosphoglycoprotein
DMP1 – Dentine Matrix protein	MGP – matrix gla protein
DNA – deoxyribonucleic acid	MHC – major histocompatibility complex
ECM – extracellular matrix	MV – matrix vesicles
EDS – energy dispersive spectrometry	NCPs – non-collagenous proteins
ELN – elastin	NPT2a - Solute Carrier family 34 (Sodium/Phosphate cotransporter) Member 1;SLC34A1
ENPP1 – ectonucleotide pyrophosphatase 1	NTPPPH – nucleoside triphosphate pyrophosphohydrolase
EPOS – European Prospective Osteoporosis Study	OA – osteoarthritis
ePPi – extracellular inorganic pyrophosphate	OPLL – ossification of posterior longitudinal ligament
ERAP – endoplasmic reticulum aminopeptidase 1	OPN – osteopontin
FBAT – family based associated test	PCR – polymerase chain reaction
FGF – Fibroblast growth Factor	PCR-SSO – polymerase chain reaction -vsequence specific primers
FPN1 – ferroportin 1	

LIST OF ABBREVIATIONS (Cont.)

PHEX - Phosphate-regulating gene with homologies to endopeptidases on the X chromosome

Pi - inorganic pyrophosphate

PSACH - pseudoachondroplasia

PTH - parathormone

PTHrP - parathormone related peptide

PTPN22 - protein tyrosine phosphatase

RA - rheumatoid arthritis

RANK - tumor necrosis factor receptor superfamily member 11a

RFC2 - replication factor C 2

RFLP - restriction fragment length polymorphism

SAMD3 -sterile alpha motif domain containing 3

SEM - scanning electron microscopy

sFRP4 - secreted frizzled related protein

SI - sacroiliac

SLC12A3 - solute carrier family 12 member 3

SLC34A1 - solute carrier family 34 member 1

SLC40A1 - solute carrier family 40 member 1

SLC9A3R1 - solute carrier family 9 isoform A3 regulatory factor 1

SNP - single nucleotide polymorphism

TDT - transmission disequilibrium test

TFR2 - transferrin receptor 2

TGF-B - transforming growth factor beta

TIBC - total iron binding capacity

TIO -tumour induced osteomalacia

VDR - vitamin D receptor

VDREs - vitamin D responsive elements

VEGF-A - vascular endothelial growth factor A

vWFA - von Willebrand Factor A

XLH - X-linked Hypophosphatemia

XRM - X-ray elemental microanalyser

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ABSTRACT

Calcification and mineralization are essential processes that result from the deposition of inorganic substances in the organic matrix of specialized tissues [1]. The terms calcification and mineralization are considered synonymous by some researchers while for others the meaning is quite diverse. Given that calcium crystal formation is the key pathological process in the disorders herein investigated - diffuse idiopathic skeletal hyperostosis (DISH) and calcium pyrophosphate dehydrate (CPPD) chondrocalcinosis (CPPD CC) - the term calcification will be used for the remainder of this thesis.

The calcification process is fundamental for the life of numerous complex and even some simple organisms. Examples include bone, tooth dentin and enamel, eggshells and exoskeletons, scales and spicules, among others. Although essential, this process is still not well understood. Multiple genes are known to be involved in the control of this process in a complex manner [2].

Detrimental calcification or mineralization can also occur, characterizing and complicating a number of pathological conditions including visceral and vascular calcifications, as well as numerous skeletal disorders. The abnormal calcification of soft tissues, termed ectopic calcification, may have various causes that range from genetic to drug related or even nutritional factors.

In recent years, the use of mouse strain models has allowed the manipulation of specific genes, and proven a powerful tool for the identification of numerous genetic determinants in ectopic calcification disorders. The improvement of strategies to study genetic mutations affecting the skeleton have also made possible the precise evaluation of the role of many different genes and proteins. The easy access to DNA/protein microarrays and to other powerful genomic techniques will surely be very important for our understanding of the molecular genetics of calcification process.

All this knowledge will hopefully not only bring new insights into the molecular mechanisms that control biomineralization in normal and pathological situations, but will also allow novel and more accurate therapeutic interventions in ectopic mineralization disorders.

The main objective of this thesis was to investigate a number of families with disorders characterised by the deposition of mineral outside the skeleton - ectopic calcification - trying to clarify the genetic basis of these pathologies. It was observed that the individuals with these conditions have their “life quality” seriously compromised due to the early onset and exuberant phenotypes. It is thus very important to determine the cause in order to discover the cure.

Two approaches have been used to achieve our goals: 1) genetic linkage study in the Azorean families (this strategy was selected given that we had access to a relatively large number of families with several generations and many affected individuals) using microsatellite markers for a whole genome wide scan analysis; 2) candidate-gene approach in chondrocalcinosis families of Slovakian origin (these families were small and the number of generations very limited). Two genes were selected for direct genomic sequencing: *ANKH* and *ENPP1*. None of the identified variants was shown to segregate with the disease. A particular family from the Azores in which Osteopoikilosis was diagnosed was selected for *LEMD3* sequencing. The disease causing mutation was identified.

The Chapter 1 of this thesis introduces the basic knowledge of what is known about the pathophysiology and genetics of ectopic mineralization (calcification and ossification), focusing on the two main disorders investigated in this thesis: DISH and CPPD-CC. Genetic and epidemiological data from both disorders will be presented in this chapter. Chapter 2 will describe the aims of this thesis and highlight the strategy that has been used. Chapter 3 will contain the detailed presentation of materials and methods. Chapter 4 will depict the results obtained from this study and Chapter 5 will contain a general discussion of the obtained data with future directions for continuation of this study.

RESUMO

A calcificação e a mineralização são processos biológicos essenciais que consistem na deposição de substâncias inorgânicas na matriz de tecidos especializados [1]. Tais termos são considerados sinónimos por alguns investigadores, enquanto para outros o seu significado é substancialmente diferente. Dado que a formação de cristais de cálcio é o processo patológico fundamental nas doenças aqui investigadas – hiperostose idiopática difusa do esqueleto (DISH) e condrocalcinose por pirofosfato de cálcio – o termo calcificação será o utilizado durante a exposição desta tese.

O processo de calcificação é fundamental para a existência de numerosos organismos complexos e mesmo até de alguns organismos simples. Exemplos da sua importância incluem o osso, a dentina e o esmalte, a casca do ovo, os exosqueletos, as escamas e as espículas, entre outros. Embora essencial, este processo não é ainda totalmente conhecido. Sabe-se, no entanto, que inúmeros genes estão envolvidos no seu controle de uma forma assaz complexa [2].

Ocorrem, frequentemente, processos de calcificação com resultados nefastos, processos estes que afectam numerosas patologias, incluindo calcificações viscerais e vasculares, bem como numerosas displasias esqueléticas. A calcificação anormal de tecidos moles é denominada como calcificação ectópica e poderá ter diferentes causas, as quais variam entre factores genéticos, mecânicos, bioquímicos e nutricionais.

Os progressos estratégicos, ao nível da genética, bem como a utilização de modelos animais, permitiram a manipulação de genes específicos, tendo-se mostrado muito útil para a identificação de numerosas determinantes genéticas de doenças caracterizadas por calcificações ectópicas. O acesso facilitado a *microarrays* e a outras poderosas técnicas do âmbito da genómica, tem-se mostrado igualmente essencial para a compreensão da genética do processo de calcificação.

Este progresso não só trará novos dados sobre os mecanismos moleculares que controlam a biomineralização em situações normais e patológicas, como também permitirá o desenvolvimento de novas, e mais eficazes, intervenções terapêuticas, em doenças caracterizadas por calcificações ectópicas.

O objectivo principal do trabalho foi, por conseguinte, o de proceder ao estudo de um conjunto de famílias com doenças caracterizadas por calcificações ectópicas, procurando esclarecer a base genética destas patologias. Foi observado que os indivíduos com tais alterações vêem a sua qualidade de vida seriamente comprometida, devido a fenótipos exuberantes. Torna-se assim muito importante determinar a causa para descobrir a cura.

Para atingir os objectivos foram então utilizadas as seguintes estratégias: 1) estudo de *linkage* em famílias açorianas (esta estratégia foi seleccionada por ser possível o acesso a um número substancial de famílias, com várias gerações e muitos indivíduos afectados;) foram utilizados marcadores genéticos – microsatélites – em todos os cromossomas autossómicos; 2) sequenciação directa de genes-candidatos em indivíduos, de origem eslovaca, diagnosticados com condrocalcinose familiar (estas famílias são de reduzida dimensão sendo o número de gerações muito limitado). Foram seleccionados dois genes para sequenciação directa: *ANKH* e *ENPP1*. Nenhuma das variantes identificadas segregava com a doença. Numa família, proveniente dos Açores, em que foi diagnosticado osteopoiquiose, procedeu-se à sequenciação do gene *LEMD3*. A mutação patogénica foi identificada.

No capítulo 1 aborda-se o conhecimento básico sobre a fisiologia e genética de mineralizações ectópicas (calcificação e ossificação), salientando as duas doenças investigadas na tese: 1) hiperostose idiopática difusa do esqueleto (DISH); 2) condrocalcinose por pirofosfato de cálcio (CPDD). Dados genéticos e epidemiológicos de ambas as doenças são apresentados neste capítulo. No capítulo 2, apresentar-se-ão os objectivos da tese. No capítulo 3 proceder-se-á à descrição dos materiais e métodos utilizados. O capítulo 4 exporá os resultados obtidos. O capítulo 5 conterà uma discussão dos dados obtidos com sugestões para estudos futuros, com vista à continuação desta investigação.

RÉSUMÉ

La calcification et la minéralisation sont des processus essentiels qui résultent de la déposition de substances inorganiques dans la matrice organique de tissus spécialisés [1]. Les termes calcification et minéralisation, sont considérés synonyme par quelques investigateurs, pendant que pour d'autres le sens est tout à fait divers. Parce que la formation de cristal de calcium est le processus pathologique primordial dans les désordres en ceci examinés – la maladie de Forrestier (DISH) et Chondrocalcinose articulaire par pyrophosphate de calcium dihydraté (CPDD) – le terme calcification sera utilisée pour le reste de cette thèse.

Le processus de calcification est fondamental pour la vie de nombreux organismes complexes et même quelques organismes simples. Les exemples incluent le tissu osseux, les dents, les coquilles d'oeuf et les exosquelettes, les échelles et spicules, parmi d'autres. Bien que essentiel, ce processus n'est pas toujours compris. Multiples gènes sont impliqués dans son contrôle d'une façon très complexe [2]. La calcification nuisible peut aussi parvenir, caractérisant et compliquant un nombre de conditions pathologiques, ceux compris calcifications viscérales et vasculaires, de même que nombreux désordres squelettiques. La calcification anormale de tissus doux, nommé la calcification ectopique, peut avoir de diverses causes qui incluent facteurs génétiques, mécaniques, biochimiques et même nutritifs.

Récemment, l'utilisation de modèles animaux a permis la manipulation de gènes spécifiques, très utile pour l'identification de nombreux déterminants génétiques des désordres caractérisé par calcification ectopiques. L'amélioration de stratégies pour étudier des mutations génétiques affectant le squelette a fait aussi possible l'évaluation précise du rôle de beaucoup de différents gènes et protéines. L'accès facile à microarrays et autres techniques puissantes de génomique sera sûrement très important pour la compréhension de processus de calcification. Toute cette connaissance amènera, non seulement de nouvelles perspicacités dans les mécanismes moléculaires qui contrôlent biomineralization dans les situations normales et pathologiques mais, aussi y permettra le développement d'interventions thérapeutiques plus précises dans les désordres de minéralisation extra-utérines. L'objectif principal de cette thèse était d'examiner un nombre de familles avec les désordres caractérisés par la déposition de minéral hors du squelette - la calcification ectopique – essayant de

clarifier la base génétique de ces pathologies. Il a été observé que les individus avec ces conditions ont leur qualité de vie sérieusement compromis dû aux phénotypes exubérants. C'est ainsi très important de déterminer la cause pour découvrir le traitement.

Deux approches ont été utilisées pour atteindre nos objectifs : 1) l'étude de liaison génétique dans les familles Azoreans; cette stratégie a été choisie parce que nous avons eu accès à un appréciable nombre de familles (12), plusieurs avec 3 générations et nombreux individus affectés qui pourrait être facilement caractérisé à cause de la dimension et magnitude de l'île. Les marqueurs génétiques utilisées, microsatellites, ont été analysés dans le génome entier, 2) l'approche de Candidat-Gène dans les familles de chondrocalcinosis d'origine slovaque; ces familles étaient plutôt petites, en comparant aux familles d'Azorean, et le nombre de générations très limité. Deux gènes ont été choisis pour séquençage direct: *ANKH* et *ENPP1*. Aucun des variantes identifiées a été associé avec la maladie. Le gène *LEMD3* a été séquencé dans une famille Azorean dans lesquelles Osteopoikilosis a été diagnostiqué. La mutation que cause la maladie a été identifiée.

Le chapitre 1 de cette thèse introduit la connaissance fondamentale de la pathophysiologie et la génétique de minéralisation ectopique (calcification et ossification), se fixant sur les deux désordres principaux examinés dans cette thèse: Hyperostose Diffuse Idiopathique Squelettique (DISH) et CPPD Chondrocalcinosis (CPDD). Données génétiques et épidémiologiques des deux désordres seront présentées dans ce chapitre. Le chapitre 2 décrira les objectifs de cette thèse et souligne la stratégie qui a été utilisée. Le chapitre 3 contiendra la présentation détaillée de matériels et les méthodologies utilisées. Le chapitre 4 décrit les résultats obtenus de cet étude et le chapitre 5 contient une discussion générale des données obtenues avec les directions futures pour la continuation de cette étude.

1. INTRODUCTION

Ectopic calcification is defined as the inappropriate mineralization of soft tissues. This process is responsible for a significant number and variety of disorders characterised by the extraskkeletal deposition of calcium and phosphate crystals [3]. Crystal deposition is, as expected, a very complex process ruled by numerous factors. Calcium and phosphate are main players in this process because they are tightly connected to the most important calcification process - bone mineralization - in which they participate directly in the formation of hydroxyapatite (HA) crystals.

Unlike calcium, phosphate homeostasis is still very poorly understood. It was only until recently that a broad number of phosphate regulators were discovered. Homeostasis regulators are expected to maintain the extracellular concentrations and equilibrium of these ions as constant as possible, in order to protect the organism against deficiency or overload. Failure of calcium and/or phosphate homeostasis can, as discussed in the following chapters, turn out to be highly detrimental. The knowledge of calcium and phosphate metabolism is thus vital to understand the basics of the ectopic calcification process.

1.1 MINERAL HOMEOSTASIS

1.1.1 Calcium homeostasis

Calcium, similarly to what is seen with other elements, exists in three different forms: ionized (50%), protein bound (40%), and complexed (10%). The ionized form is the one that interests us most because it is the free, usable form. On a broad view, circulating calcium and phosphate concentrations are maintained by a dynamic equilibrium through fluxes occurring at the level of:

- i. the intestine (absorption through cellular mechanism and paracellular mechanism),
- ii. kidney (central role in regulating the extracellular concentration of either ion; excreted by filtration and reabsorption with active cellular transport and paracellular diffusion),
- iii. and bone (exchange of mineral by cellular bone resorption and possible diffusion through bone cell envelope) [4].

Control of calcium metabolism is controlled by systemic regulating hormones including parathyroid hormone (PTH), 1,25-dihydroxyvitamin D (calcitriol) and calcitonin. Many other factors play roles on calcification such as: thyroid and pituitary hormones, adrenal and gonadal steroids, cytokines, and growth factors [5].

PTH is encoded on chromosome 11. When calcium levels drop, PTH is secreted, leading to increased calcium absorption through the gut, an increase in calcium reabsorption through the kidney, and an increase in bone resorption through direct and indirect processes. The effect of PTH on calcium renal absorption is direct and is performed by means of a calcium sensing receptor - CASR. CASR is a plasma membrane G protein-coupled receptor that is expressed in the parathyroid hormone-producing chief cells of the parathyroid gland and the cells lining the kidney tubule. Because this protein can sense small changes in circulating calcium concentration it plays an essential role in maintaining mineral ion homeostasis [6]. Intestinal absorption of calcium is indirectly mediated by

PTH via the stimulation of the 1α hydroxylation of 25OHD to produce $1,25(\text{OH})_2\text{D}$, which is the direct mediator of calcium intestinal reabsorption.

Vitamin D can be synthesized as vitamin D3 (cholecalciferol) under the influence of ultraviolet light within the skin, or ingested, either as vitamin D3 or D2 (ergocalciferol). It is subsequently transported to the liver by a binding protein where it undergoes 25-hydroxylation; 25-hydroxyvitamin D (25OHD) is then hydroxylated in the 1 position by the renal 1α hydroxylase. $1,25(\text{OH})_2\text{D}$ is the active metabolite of vitamin D which promotes the synthesis of a calcium transporting protein within the cells of the small intestine. Bone is also a target organ for this metabolite. The effects of $1,25(\text{OH})_2\text{D}$ are mediated through a widely distributed nuclear vitamin D receptor (VDR) which contain DNA and hormone binding motifs. VDR is a phosphoprotein that regulates gene expression by heterodimerizing with retinoid X receptor and associating specifically with vitamin D responsive elements (VDREs) in target genes [7].

Calcitonin is a 32-amino-acid peptide synthesized as a precursor peptide in the C cells of the thyroid. Its secretion is increased by an increase in plasma calcium. The main effect of calcitonin is to reduce bone resorption by direct and reversible suppression of specific G-protein-linked receptors on the osteoclast. The physiological role of calcitonin remains uncertain despite intensive research in the topic [4].

Parathyroid hormone related protein (PTHrP) is encoded by a gene located in the short arm of chromosome 12. It is very similar to PTH in terms of effects and sequence. There are three isoforms of PTHrP resulting from alternative splicing; each isoform has different effects. Gene expression is controlled by three different promoters which are regulated by agents such as TGF- β and glucocorticoids. PTHrP was originally detected in numerous tumours particularly in squamous cell carcinomas and lung where its secretion causes humoral hypercalcemia of malignancy [4]. Its physiological role is in control of cartilage proliferation and differentiation and thus endochondral ossification. Whilst PTH is a circulating hormone carrying signals from a calcium sensor in the parathyroid glands to remote target tissues, PTHrP is a polyhormone with functions of local messenger within tissues [8].

1.1.2 Phosphate homeostasis

Intracellular phosphate has a structural role in nucleic acids, phospholipids and phosphoproteins; it forms high-energy phosphate bonds and is involved in cellular signalling [9]. The majority of extracellular phosphate can be found as bone mineral. The main determinant of plasma phosphate concentration is the maximal renal tubular reabsorption of phosphate (TmP/GFR) [10]. It was, until recently, scientifically accepted that regulators of calcium homeostasis, PTH, Vitamin D and calcium sensing receptors, passively mediated phosphate homeostasis. However, recent studies have suggested a new, more complex, regulatory pathway for phosphate balance, partially independent from calcium homeostasis. Two new groups of factors, the “phosphatonins” and “minhibins”, have emerged as the major regulators of phosphate homeostasis [11, 12], besides the classically accepted PTH and Vitamin D.

The role of $1,25(\text{OH})_2\text{D}_3$ in bone turnover and phosphate homeostasis is to promote intestinal Ca^{2+} and Pi absorption and, in cooperation with PTH, induce the expression of macrophage colony stimulating factor and RANK ligand by osteoblasts, stimulating bone catabolism and resorption. Control of the phosphatemic/calcemic effects of vitamin D is therefore essential. Part of this control is performed by the effect of PTH:

1) on renal phosphate reabsorption (through the internalization of NaPi-IIa/IIc transporters);

2) by its control of 1α hydroxylation.

Studies of two hypophosphatemic disorders (autosomal dominant hypophosphatemic rickets – ADHR - and the X-linked Hypophosphatemia), and of tumour induced osteomalacia (TIO), have identified respectively a fibroblastic growth factor-23 (FGF23) [13], a cell surface metalloprotease (PHEX) [14], and the matrix extracellular phosphoglycoprotein (MEPE) [15]. These proteins were shown to constitute a hormone/enzyme/extracellular matrix axis that regulates phosphate homeostasis [16, 17].

Table 1 Summary of central proteins on phosphate homeostasis:

Gene	Name	Function of protein	Ref
NPT2a	Solute carrier family 34 (sodium/phosphate cotransporter) member 1;SLC34A1	Phosphate transepithelial transport across the proximal renal tubule is mediated by type II family of transporters that include NaPi-IIa (NPT2a and SLC34A1), NaPi-IIb (SLC34A2) and NaPi-IIc (SLC34A3). This transport is the responsible for keeping serum concentration of Pi stable at 1.1.mM. The first group NaPi-IIa, is responsible for ~85% of renal Pi reabsorption. Small alterations in the expression of this gene or in the activity of its protein have significant consequences in Pi homeostasis and phosphaturia. NaPi-IIa knockout mice show a phenotype compatible with hypophosphatemic rickets with hypocalcemia. No mutations were found on humans with this disorder, though. Mutations in the other Type II family of transporter genes results in urolithiasis, osteoporosis and pulmonary alveolar microlithiasis [18, 19].	[19]
sFRP4	Secreted frizzled-related protein 4	This protein is a member of the SFRP family that contains a cystein-rich domain homologous to the putative wnt-binding site of frizzled proteins. SFRPs act as soluble modulators of wnt signaling. Has phosphaturic effects by specifically inhibiting sodium-dependent phosphate uptake.	[20, 21]
FGF23	Fibroblast growth factor 23	Member of the fibroblast growth factor (FGF) family. Full length FGF23 is the active form, inhibits phosphate uptake and mineralization <i>in vivo</i> by suppressing expression of type IIa and IIc sodium/phosphate cotransporters in the brush border membranes of proximal tubes. It also reduces serum 1,25(OH) ₂ D ₃ by modifying expression levels of the vitamins D-metabolizing enzymes. Cleaved FGF23 is inactive.	[13]
PHEX	Phosphate-regulating gene with homologies to endopeptidases on the X chromosome	Type II, zinc dependent, transmembrane endopeptidase involved in bone and dentin mineralization and renal phosphate reabsorption. PHEX protein binds to MEPE protecting it from the proteolytic release of the ASARM peptide (see below).	[14]
Klotho	Co-factor of FGF23	Klotho is a single pass transmembrane protein with beta-glucuronidase activity. Klotho binds directly to FGF receptors and the Klotho-FGF complexes bind to FGF-23 with much higher affinity. So it has been suggested that Klotho is an essential cofactor for the FGF23 signalling. Reduced production of this protein has been observed in patients with chronic renal failure (CRF), and this may be one of the factors underlying the degenerative processes (e.g., arteriosclerosis, osteoporosis, and skin atrophy) seen in CRF. Also, mutations within this gene have been associated with ageing and bone loss in mice. The Klotho peptide generated by cleavage of the membrane-bound isoform may be an anti-aging circulating hormone which would extend life span by inhibiting insulin/IGF1 signaling.	[22]
MEPE	Matrix extracellular phosphoglycoprotein	Several studies have indicated MEPE as a mineralization inhibitor mainly because it contains an acidic-serine-aspartate rich	[15]

			associated motif (ASARM) consisting on the last 18-C terminal amino acids of the MEPE protein. This motif is suggested to play an important role in preventing calcification of the urinary tract as well as inhibiting precipitation of calcium/Pi in supersaturated saliva solution.	
DMP1	Dentine matrix protein 1		DMP1 is another acidic phosphorylated matrix protein, similar and from the same family as MEPE (SIBLING). Proteolytic degradation of DMP1 leads to the release of the ASARM peptide with potent minihitin and phosphatonin-like activity.	[23]
ASARM	Acidic serine- and aspartate-rich associated motif (ASARM) peptides	MEPE-	The ASARM peptide inhibits mineralization directly by binding to hydroxyapatite crystals and decreased expression of PHEX, whereas phosphaturic effects are mediated by renal accumulation of ASARM peptides and inhibition of Pi reabsorption.	[24]

1.1.3 Inorganic pyrophosphate homeostasis

Inorganic pyrophosphate (PPi), which consists of two molecules of inorganic phosphate (Pi) connected by a hydrolyzable high energy ester bond, is a key factor involved in regulation of crystal deposition [25]. Pi is an essential element for the physiological functioning of osteoblasts, not only because it is an integral component of apatite crystal but also because it can affect the production rate of the bone matrix [26]. For normal mineral deposition to proceed the ratio of extracellular Pi and PPi is required to be in tight balance. PPi production and its elaboration into the extracellular space is a unique property of chondrocytes. Extracellular PPi (ePPi) is required to induce calcification and is a potent inhibitor of crystallization of Pi with calcium to form hydroxyapatite thereby suppressing hydroxyapatite (HA) crystal propagation [27, 28].

PPi is produced, degraded and transported by specialized mechanisms. Deregulation of any of these processes has been associated with disease, such as calcium pyrophosphate deposition disease (CPDD), in which PPi appears to mediate specific disease manifestations [25].

PPi is produced in considerable amounts either indirectly, as a metabolic byproduct of multiple biochemical and biosynthetic reactions during synthesis and secretion of matrix proteins (especially collagen), or directly by ectoenzymes that generate Pi (pyrophosphatases and ATPases) and PPi (nucleoside triphosphate pyrophosphohydrolase - NTPPPH) on apoptotic particles and MV membrane surfaces [25, 29, 30]. The major cloned enzymes with NTPPPH activity

in cartilage are plasma cell glycoproteins (PC-1). Extracellular PPI (ePPI) is generated by these enzymes and secreted along with newly synthesized collagen [29, 30]. To prevent toxic accumulation, there must be an efficient way of disposing the PPI as it is produced. Ubiquitous pyrophosphatases are thought to be responsible for this high turnover and for maintaining generally low intra- and extracellular concentrations of PPI [25, 26].

Although PPI does not passively cross cell membranes, it can become compartmentalized in cells and matrix vesicles by active and facilitated transport mechanisms [26]. Recent studies have clarified the role of the multiple-pass membrane protein ANK in mammalian cells as a mediator for PPI channelling to the cell exterior. This pathway clearly regulates tissue calcification and susceptibility to arthritis by influencing both intracellular and extracellular PPI concentrations [31]. The three major regulators of pyrophosphate homeostasis can be seen in the next table.

Table 2. Pyrophosphate main regulators

Function	Name	Gene and Protein
Breakdown of ATP / NTPs	NTPPH (e.g. ENPP1)	Ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) is an ubiquitous cell surface membrane bound glycoprotein with enzymatic activity. It produces inorganic pyrophosphate from ATP, inhibiting pyrophosphate related calcification and mineralization. <i>ENPP1</i> maps to chromosome 6 (6q22-q23) and comprise 25 exons that encode a protein expressed in a variety of tissues including matrix vesicles of bone and cartilage.
Breakdown of PPI	TNAP	Tissue non specific alkaline phosphatase (TNAP) is an isozyme of a family of four homologous human alkaline phosphatase genes. It is present in the matrix vesicles and has the ability of hydrolyze PPI. It is encoded by <i>TNAP</i> which maps to chromosome 1 and contains 12 exons.
Transport of PPI	ANKH	<i>ANKH</i> encodes a 492 amino acid multiple-pass transmembrane protein which is involved in the transport of inorganic pyrophosphate (PPI) across plasma membrane to the extra cellular compartment. Mutations in <i>ANKH</i> have already been associated with a ectopic calcification disorder characterised by the deposition of calcium pyrophosphate crystals in the cartilage [32].

1.2 MINERAL DEPOSITION

1.2.1 Organelles - matrix vesicles

Mineral deposition is a tightly controlled process both in terms of tissue specificity and rate. The key organelles in this process are chondrocyte and osteoblast matrix vesicles (MVs), in which the principal calcium/phosphate crystals, consisting primarily of calcium hydroxyapatite (HA) or calcium pyrophosphate dihydrate (CPPD), are formed [[33],[34],[35],[36]]. Matrix vesicles bud away from the plasma membrane of the osteoblasts or chondrocytes to extracellular space, where they serve as small packages of mineral and as mineralisation niduses [37]. MVs are extracellular membrane-invested organelles with 50-200 nm in diameter. The first crystals of calcium HA mineral are generated within MVs during biomineralization of growth plate cartilage, newly formed bone, tendon and the predentine of teeth [38].

The molecular components of MVs include:

- 1) lipids – typical plasma membrane phospholipids (e.g. cholesterol and sphingomyelin) and an unusual high concentration of acidic phospholipids (e.g. phosphatidylserine and phosphatidic acid) [39];
- 2) alkaline phosphatase – this enzyme, also known as “Tissue non-specific alkaline phosphatase” (TNAP) is characteristically enriched in MVs where it concentrates at the outer surfaces of the MV membrane; this association is essential for calcium deposition by isolated MVs [39];
- 3) other phosphatases – adenosine monophosphoesterase (AMPase), nucleotide pyrophosphatase (e.g. ENPP1) [39], ATPases [40] and a new phosphatase designated Phospho1 [41], were shown to be present in calcifiable MVs;
- 4) Non-phosphatase proteins such as Annexin V [42], bone sialoprotein, osteonectin, and osteocalcin are calcium binding matrix proteins of the bone that were shown to play a role in mineralisation [43].

The calcification process, initiated by MVs, starts with the absorption of Ca^{2+} driven by the high concentration of Ca-phospholipids and proteins and also due to the high activity of Annexin V. Meanwhile, PO_4^{3-} accumulates within the MV as a

result of PO_4^{3-} transport by the Na-PO_4 transporter plus enzymatic activity of phosphatases. When sufficient Ca^{2+} and PO_4^{3-} have accumulated in the interior of the MVs, amorphous CaPO_4 starts to precipitate. This non-crystalline form will be converted to octacalcium phosphate which is then transformed into highly insoluble hydroxyapatite [44].

Once initiated, this process seems to be a cascade, which is stimulated by crystals themselves, and inhibited by certain inhibitor molecules, like pyrophosphate or acidic non-collagenous proteins (NCPs) [37].

Matrix vesicles have other important roles besides mineralization. They contain significant quantities of bone morphogenetic proteins (BMPs). They also regulate the digestion of proteoglycans, components of growth plate cartilage matrix, due to the presence of matrix metalloproteinases and peptidases [38].

1.2.2 Cells - chondrocytes

The chondrocytes are cartilage-forming cells that differentiate from multipotential bone marrow mesenchymal stem cells by a multipotential pathway known as chondrogenesis [45]. Chondrocytes are the main constituents of tissues affected by chondrocalcinosis and are the main cell lines used to investigate disorders such as CPPD-CC. The knowledge of chondrocytes structure, function and differentiation will surely add useful information to the studies that should be undertaken to investigate ectopic calcifications in cartilage.

The chondrocyte cytoskeleton is a three-dimensional network composed of three types of protein networks: actin microfilaments, microtubules and intermediate filaments. Microfilaments have been clearly implicated in control of chondrocyte phenotype being important in cell-matrix interactions, cell-signaling, differentiation, intracellular transport, control of secretion/endocytosis and in resiliency of cell shape. Microtubules have been associated with intracytoplasmic transport and intermediate filaments with mechanical as well as transport and signalling functions [46].

Chondrogenesis is a fundamental process in vertebrates since it leads to the formation of cartilage growth plates and has a major role in endochondral ossification. Thus, chondrocytes are vital components of the skeleton

contributing decisively for the osteogenesis and angiogenesis. They are the predominant cell type in growing cartilage and their main function is to synthesise the cartilage matrix through which nutrients and waste materials are exchanged between the bloodstream and the cells [47].

During endochondral ossification chondrocytes express characteristic cartilaginous matrix genes including type II collagen, type IX collagen, type XI collagen, aggrecan, chondromodulin-1 and matrilin-3, among others [48]. A specific type of collagen (type X) is synthesised by chondrocytes after they have become hypertrophic and before mineralisation of the extracellular matrix occurs [49]. The primary mechanisms for the linear growth of bones are chondrocyte proliferation and matrix elaboration in the direction of bone growth and the hypertrophy of these cells are [4]. Chondrocytes mineralise the intercolumnar matrix producing a rigid scaffold, which persists into the metaphyses and becomes the solid base upon which osteoblasts deposit and mineralise bone matrix.

Chondrocyte proliferation, differentiation and apoptosis is regulated by numerous factors including Fibroblast Growth Factors (FGFs) [50], Bone Morphogenetic Proteins (BMPs) [51], Insulin like Growth Factor 1 (IGF-1) [52] PTHrP [53], extracellular matrix components [54], biomechanical signals [55], and members of the hedgehog [56] and Wnt families [57, 58]. Transduction of all these signals within the developing mesenchymal cells and chondrocytes results in changes in gene expression mediated by several transcription factors, including Sox9 [59, 60], and Cbfa1, leading to chondrocyte differentiation [61, 62].

1.2.3 Tissues – cartilage and enthesis

Tissues affected by the disorders under study in this thesis will be briefly introduced. CPPD-CC and DISH affects fibrous and hyaline cartilage, paravertebral ligaments and enthesis.

1.2.3.1 Cartilage

Articular cartilage is composed of a relatively small number of cells – chondrocytes – surrounded by a multicomponent matrix containing water, proteoglycans and collagen.

Chondrocytes comprise ~5% of the tissue volume and control the ability to synthesize and degrade the matrix.

Approximately 70 to 85% of the weight of the whole tissue is water.

Approximately 30% of the dry weight is composed of proteoglycans (PGs). Proteoglycan is a molecule where at least one glycosaminoglycan side chain is covalently attached to the protein core of the molecule forming a bottle-brush-like structure [63]. Proteoglycans are negatively charged, and are trapped within the collagen mesh attracting cations (mainly Na⁺) and water. This causes PGs to swell, increasing the tension within the collagen network until a steady state is reached giving the tissue the ability to resist compression [64].

Aggrecan, the major load-bearing proteoglycan in the extracellular matrix of all cartilaginous tissue, is composed of a ~300kDa core protein substituted with ~100 chondroitin sulphate (CS) and, in some species, keratan sulphate (KS) glycosaminoglycan (GAG) chains. Aggrecan is a member of the hyaluronan-binding proteoglycan family (which also includes brevican, neurocan and versican) and associates noncovalently with hyaluronan and the ~45kDa link glycoprotein to form high molecular weight aggregates [65]. Although the aggrecan aggregate is the major component of cartilage ECM, versican/PG-M is also present in cartilage but at low levels. Versican/PG-M is a large chondroitin sulfate proteoglycan of the extracellular matrix (ECM), with a common domain structure to aggrecan [66].

Approximately 1 to 10% of the cartilage glycosaminoglycans are composed of hyaluronan which has diverse and important biological roles in vertebrates. These roles include: acting as a vital structural component of connective tissues, formation of loose hydrated matrices that allow cells to divide and migrate, immune cell adhesion and activation, and a role in intracellular signalling. All this diversity results from the large number of hyaluronan-binding proteins (often called hyaladherins), that exhibit significant differences in their tissue expression,

cellular localization, specificity, affinity and regulation [67]. The ubiquitous hyaluronan receptor CD44 is known to participate in a wide variety of cellular functions including the cell-cell aggregation, matrix-cell and cell-matrix signalling, organization and turnover of extracellular matrix at the cell surface, receptor-mediated internalization/degradation of hyaluronan, cell migration and mediates the migration of lymphocytes during inflammation [68].

Another important constituent of articular cartilage is the cartilage oligomeric matrix protein (COMP), a member of the thrombospondin family of extracellular matrix glycoproteins [69]. It has been proposed that COMP molecules are important for maintaining the properties and integrity of the collagen network [70] and contribute to the material properties of biological tissue [71]. Mutations in COMP have been associated with two dominantly inherited skeletal disorders: pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED) [72].

Matrillins are a subfamily of extracellular matrix proteins containing the von Willebrand Factor A (vWFA)-like domains. This family of fibril-forming vertebrate ECM proteins contains 2 vWFA domains, which are involved in cell adhesion, in extracellular matrix proteins, and in integrin receptors [73, 74].

Collagen is a fibrous protein that makes up 60 to 70% of the dry weight of the tissue. Type II is the predominant collagen of articular cartilage although other collagen types (e.g. type XI) are present in smaller amounts. Collagens give the tissue the ability to resist tension defining the shape and form of the tissues in which they occur [64]. The fibrils are arranged in elaborate 3-dimensional arrays, such as parallel bundles (e.g. in tendons and ligaments), orthogonal lattices (e.g. in cornea) and concentric weaves (e.g. bone) and are synthesized by fibroblasts by a poorly understood process [75].

Articular cartilage is typically depicted in 4 areas between the articular surface and the subchondral bone: the surface or superficial tangential zone, the intermediate or middle zone, the deep or radiate zone and the calcified zone. Each area has its own distinct matrix region with differences observed in the collagen structure [63, 64].

Three types of cartilage can be found in human body: hyaline, elastic and fibrous [76]:

- 1) Hyaline cartilage, the most abundant type, covers the ends of bones, where bones come together, to form joints. It is found in the growth plates of bones and the embryonic skeleton. Solid matrix with small and evenly dispersed collagen type II fibers throughout the ground substance making the matrix appear transparent; chondrocytes are found in the lacunae.
- 2) Fibrocartilage is found in intervertebral disks, symphysis pubis, knee and temporomandibular joints. It is structurally similar to hyaline cartilage, but in addition to type II collagen fibers, which are more numerous, type I collagen is also present and is arranged in thick bundles.
- 3) Elastic cartilage is found in the external ear, epiglottis and auditory tube. The structure is similar to hyaline cartilage, but the matrix also contains abundant elastic fibers scattered through the matrix.

1.2.3.2 Entheses

An enthesis is an attachment or insertion site. It is the region where a tendon, ligament or joint capsule attaches to bone. They can either be fibrous or fibrocartilaginous according to the tissue at the bone-tendon interface; dense fibrous connective tissue or fibrocartilage, respectively [77,78].

Anatomically the enthesis can be divided into 4 histological areas that merge gradually with each other [79]:

1. Tendon or ligament: band of collagen fibers that possess considerable strength and are able to resist traction;
2. Unmineralized fibrocartilage: between tendon and bone is a zone of cartilage which varies in thickness depending upon the specific tendon and consisting of collagen fibers similar to those of the tendon and chondrocytes;
3. Mineralized fibrocartilage: abrupt transition from the previous area. Crystals become apparent between and within the collagen fibrils.
4. Bone: collagen fibers on the mineralized fibrocartilage blend with those of the bone matrix.

Alterations at enthesis, enthesopathies, are common and characteristic of many disorders, including traumatic, degenerative, inflammatory, endocrine and metabolic conditions [79]. Abnormalities of entheses are characteristic of some crystal-induced disorders such as CPPD crystal deposition disease (CPDD), HA crystal deposition disease and alkaptonuria [80]. DISH manifestations include numerous enthesopathic hyperostosis including bone outgrowths at attachments of tendons and ligaments [81].

Besides enthesopathic calcifications, in CPDD disorder, calcification may occur in tendons (supraspinatus) and ligaments (achilles, triceps and quadriceps). Capsular calcification can also occur, especially in the elbow and metatarsophalangeal articulations [80]. In DISH it can be seen ligamentous calcification and ossification and para-articular bridging osteophytes in the anterolateral aspect of the spine, particularly in the lower thoracic region, and in extraspinal sites such as the pelvis, calcaneus, patella and olecranon [81].

1.3 MOUSE MODELS SHOWING ECTOPIC CALCIFICATION

1.3.1 *ank* mouse

Mice carrying the “progressive ankylosis” mutations have been studied as a model of arthritis.

This mouse model showing a recessive skeletal mutation causing noninflammatory joint disease with progressive ankylosis, *ank*, was first described by Sweet and Green [82]. The authors described three main abnormalities:

- 1) increased calcification of calcified cartilage and of joint tissues,
- 2) hyperplasia of the cells and tissues of the joints, and
- 3) degeneration within and around the tissues, tendons, and ligaments of the joints.

In detail, the autosomal recessive mutation causes the loss of mobility of most joints throughout the limbs and vertebral column leading to complete rigidity and death around the 6 months of age.

The formation of hydroxyapatite crystals in articular surfaces, joint space narrowing, cartilage erosion and formation of osteophytes which cause fusion and joint immobility, mimic several human diseases such as chondrocalcinosis, osteoarthritis and ankylosing spondylitis [82, 83].

This locus was mapped to mouse chromosome 15 [82]. Ho et al., some years later, identified the mutation in the mouse *ank* gene and showed that it encodes a multiple-pass transmembrane protein involved in the regulation of pyrophosphate levels in cultured cells. By radiation hybrid mapping this group mapped the human *ANK* gene to chromosome 5p [84].

Mutations in *ANKH* were shown to cause the autosomal dominant conditions: chondrocalcinosis [32] and craniometaphyseal dysplasia (CMD) [85].

1.3.2 *ttw* mouse

This is naturally occurring mouse model, first described by Okawa et al. [86]. *Ttw* mouse phenotype resembles the human condition: ossification of the posterior longitudinal ligament (OPLL). This mouse also presents hydroxyapatite chondrocalcinosis.

Using a positional candidate-gene approach, this group determined that the *ttw* phenotype is caused by a nonsense mutation in the *ENPP1* gene which encodes the enzyme nucleotide pyrophosphatase. This enzyme, as previously described, regulates soft-tissue calcification and bone mineralization by producing inorganic pyrophosphate.

Mutations in *ENPP1* were shown to cause the autosomal recessive condition idiopathic infantile arterial calcification (IIAC) [87]. *ENPP1* mutations may be associated with insulin resistance in individuals with type II diabetes mellitus [88].

1.3.3 *Akp2* mouse

Narisawa et al. reported in 1997 the inactivation, by homologous recombination, of two of the three active mouse alkaline phosphatase genes (embryonic - EAP, and tissue-non specific - TNAP). Whereas EAP knock-out mice show no obvious phenotypic abnormalities, TNAP knock-out mice mimic a severe form of hypophosphatasia with growth impairment, epileptic seizures, apnea and death before weaning. Tissue examination revealed abnormal bone mineralization, osteoblasts with anomalous morphology, aberrant development of lumbar nerves, disturbances in intestinal physiology, increased apoptosis in the thymus and abnormal spleens [89].

The biochemical hallmark of hypophosphatasia is subnormal alkaline phosphatase activity in serum (hypophosphatasemia), leading to elevated blood or urine levels of three phosphocompounds: phosphoethanolamine (PEA), inorganic pyrophosphate (PP_i), and pyridoxal 5'-phosphate (PLP). An interesting study, reported recently, revealed that enzyme replacement using a bone-targeted, recombinant form of human TNAP prevents infantile hypophosphatasia in *Akp2*^{-/-} mice [90].

1.3.4 *Kl* mouse

Klotho mutant mice (*kl/kl* mice), were first described in 1997. They manifest a syndrome that resembles human ageing, including a short lifespan, infertility, arteriosclerosis, skin atrophy, ectopic calcification, increases in serum calcium and phosphorus, osteoporosis and emphysema [91].

Klotho contains 5 exons and spans approximately 50kb of DNA [92]. It encodes a membrane protein that shares sequence similarity with the beta-glucosidase enzymes [91]. This protein may function as part of a signalling pathway that regulates ageing in vivo and morbidity in age-related diseases [91]. As previously mentioned, *Klotho* has an essential role in phosphate metabolism by binding to FGF23 [22]. A role by binding to Wnt family members suppressing their biological activities was suggested recently [93]. An important role in calcium metabolism has also been suggested [94]. Although not fully understood, observations suggest that *klotho* may participate in a negative regulatory circuit of the vitamin D endocrine system, through the regulation of 1α -hydroxylase gene expression [95].

1.3.5 *Mgp* mouse

Matrix-gla protein is a potent inhibitor of soft tissue ectopic mineralization. It is a small, 79-amino acid ECM protein that contains posttranslationally modified γ -carboxyglutamic acid residues resulting from vitamin K-dependent carboxylation of the protein in the endoplasmic reticulum [96].

The *Mgp* mice were created by disrupting one *Mgp* allele by gene targeting in embryonic stem cells. Heterozygous mice were then crossed to generate homozygous *Mgp*-deficient mice. These mice had premature death, at 2 months of age, due to arterial calcification. *Mgp* mice show inappropriate calcification of various cartilages including growth plate, which leads to short stature, osteopenia and fractures [97].

1.3.6 *Hyp* mouse

In 1976, Eicher et al. report a new mutant mouse characterized by hypophosphatemia, bone rickets, dwarfism and high excretion of phosphate [98]. The Hyp mouse is a naturally occurring mouse with a spontaneous mutation that has been bred, since its identification, on the C57BL/6J background. Like their human counterparts, this murine model has renal phosphate wasting, impaired mineralization, and growth retardation. The renal defect in phosphate reabsorption in Hyp mice is believed to reside at the level of the brush-border membrane of the proximal tubule [99]

This mouse was considered the perfect model for the X-linked hypophosphatemia (XLH). A few years later the Hyp Consortium isolated a candidate gene from the Xp22.1 region, by positional cloning. It was discovered the disease causing mutation; a large deletion in the 3-prime region of the *Phex* gene [100]. Since then, many other mutations have been associated with XLH [101].

Bone cells of this murine model have been widely studied. Recent studies have concluded that aberrant *Phex* function in osteoblasts and/or osteocytes alone is sufficient to underlie the Hyp phenotype [102].

1.4 TYPE OF ECTOPIC CALCIFICATIONS

Ectopic calcifications can be attributed to one of three mechanisms - dystrophic, metastatic or idiopathic – according to its pathogenesis. Ectopic ossification may also occur in some cases [103].

1.4.1 Dystrophic calcification

Dystrophic calcifications often occur as the result of a traumatic injury in tissues where the biochemistry values are normal. Causes for this type of calcification include inflammation, haemorrhage, increasing age and connective tissue disorders.

Table 3. Examples of dystrophic calcifications

OMIM	Disorder	Clinical phenotype	Ref.	Gene
610455	Tumoral calcinosis, Normophosphatemic, familial NFTC	Rare disorder characterised by massive periarticular and rarely visceral deposition of calcified tumors. Subset of FTC with normal values of phosphate. Examination of lesions biopsies revealed massive calcium deposition in the mid- and lower dermis.	[104] [105] [106]	<i>SAMD9</i>
181750	Scleroderma, familial progressive; Systemic sclerosis; CREST syndrome included	Clinically heterogeneous connective disorder characterized by immune activation, vascular damage and fibrosis of the skin and internal organs. Multifactorial involving both genetic and environmental factors.	[107] [108]	Unknown
611618	Arthropathy, Tendinous calcinosis and progeroid features	Syndrome involving arthropathy (affecting predominantly the distal femora and proximal tibia) , tendinous calcinosis (in the knee) and progeroid features (small pinched nose, small lips, micrognathia with crowded teeth, cataract, alopecia, lipodystrophy and sclerodermatous skin).	[109]	<i>LMNA</i>
152700	Systemic Lupus Erythematosus; SLE	Chronic, remitting, relapsing, inflammatory and often febrile multisystemic disorder of connective tissue with involvement of skin, joints, kidneys and serosal membranes.	[110] [111]	HLA Class II, <i>PTPN22</i> , and many others
173700	Poikiloderma, hereditary sclerosing	Generalized poikiloderma accentuated in flexural areas and on extensor surfaces, sclerosis of the palms and soles and in some cases late development of subcutaneous calcification.	[112] [113]	Unknown

+120940	Dermatomyositis	Uncommon disease of unknown cause that can occur in childhood (Juvenil) or in adulthood and affects predominantly females. Juvenile dermatomyositis is associated with the development of soft-tissue pathological calcification. Hydroxyapatite is the main constituent of calcification deposits. Infection agents are thought to play a role in the onset of this disorder. More than 70% of the affected individuals have the presence of HLA Class II allele DQA1*0501.	[114]	HLA-DQA
	Heterotopic ossification after hip replacement	Common complication induced by surgical trauma of soft tissues. Between 2 and 90% of the individuals that went through major hip procedures such as internal fixation of acetabular fracture and total hip arthroplasty suffer from heterotopic ossification. Proposed risk factors include AS, hypertrophic osteoarthritis, DISH, biochemical factors, male sex, age over 60 and previous of heterotopic bone formation.	[115-117]	Unknown

1.4.2 Metastatic calcification

Metastatic calcifications occur in tissues that are not damaged but in which there are abnormal biochemistry values such as high or low calcium, phosphate, oxalates, and other molecules such as iron and copper. Causes for this type of calcification include: in the presence of hypercalcaemia: hyperparathyroidism, excessive vitamin D, excessive vitamin A, therapeutic use of retinoids, neoplastic disease, and sarcoidosis. It affects blood vessels, soft tissues such as cornea, conjunctivae, tendons and ligaments and nephrocalcinosis. In the presence of hypocalcaemia the most common causes are hypoparathyroidism and pseudohypoparathyroidism Type 1A (Albright hereditary osteodystrophy; AHO). Affected tissues include basal ganglia and subcutaneous tissues. Hyperphosphataemia may be inherited or result from renal glomerular failure or parathyroid insufficiency or resistance. Affected tissues are blood vessels, soft tissues and periarticular soft tissues. When low levels of phosphate are seen the underlying cause is inherited hypophosphataemia affecting tendons and ligaments [4, 103].

Table 4. Metastatic disorders due to high phosphate levels

OMIM	Disorder	Clinical phenotype	Ref.	Gene
2111900	Familial Tumoral calcinosis, hyperphosphatemic	Progressive deposition of basic calcium phosphate crystals in periarticular spaces and soft tissues	[118, 119] [106]	<i>GALNT3</i> <i>FGF23</i> <i>KLOTHO</i>
610233	Hyperostosis-Hyperphosphatemia syndrome; HHS	Recurrent, transient, painful swellings of the long bones associated with the radiographic findings of periosteal reaction and cortical hyperostosis. Serum phosphate increased.	[120]	<i>GALNT3</i>
203330 603233 103581	Pseudohypoparathyroidism	Heterogeneous group of disorders characterized by resistance to parathyroid hormone. A large proportion of the affected individuals show subcutaneous calcifications as well as ectopic ossifications.	[121]	<i>GNAS1</i>

Table 5. Disorders with metastatic calcification due to low phosphate or low alkaline phosphatase levels:

OMIM	Disorder	Clinical phenotype	Ref.	Gene
241510	Hypophosphatasia, childhood	Inborn error of metabolism clinically characterised by defective bone mineralization and biochemically by deficient activity of the tissue-non specific isoenzyme of alkaline phosphatase. Segregation can be either autosomal dominant or recessive.	[122]	<i>ALPL</i>
241500	Hypophosphatasia, infantile	Heterogeneous disorder; often lethal especially if inherited specific alleles in homozygosity. Cases with hypophosphatasia with concurrent monoarthritis attacks with widespread calcification of articular cartilage (chondrocalcinosis) were reported. In addition to phosphoethanolamine (PEA), inorganic pyrophosphate (PPi), was elevated in plasma and in urine of some affected individuals.	[123] [124]	<i>ALPL</i>
146300	Hypophosphatasia, Adult	Heterogeneous biochemical and clinical features which include: low levels of serum alkaline phosphatase, high levels of serum pyridoxal-5'-phosphate (PLP), early loss of	[125] [126] [127] [128]	<i>ALPL</i>

		teeth (odontohypophosphatasia), bowed legs, calcification of paraspinous ligaments, joint pains and peri and intraarticular calcifications of joints of the hands, feet and knees.		
307800	Hypophosphatemic rickets, X-linked dominant	X-linked dominant disorder characterised by low levels of serum phosphate and normal or low levels of serum 1,25-dihydroxyvitamin D3. Growth retardation, rachitic and osteomalacic bone disease and renal defects in phosphate reabsorption were also observed. Some adults, especially males, may develop progressive ankylosis of the spine and major joints resembling ankylosing spondylitis. Calcification and ossification of entheses can occur in ~70% of patients. Osteoarthritis is common in ankles, wrists, knees, feet, and sacroiliac joints. Older patients have enthesopathy, often with the presence of extra ossicles. Curvatures in the lower extremity of long bones are common in all age groups.	[129] [130] [131] [132]	<i>PHEX</i>
612286	Nephrolithiasis	Patients with hypophosphatemia and decreased renal phosphate absorption.	[133]	<i>SLC34A1</i>
612287	/Osteoporosis, Hypophosphatemic, 1 Nephrolithiasis /Osteoporosis, Hypophosphatemic, 2	Recurrent urolithiasis. Spinal deformities and recurrent fractures.	[19]	<i>SLC9A3R1</i>

Table 6. Metastatic calcification due to hypercalcaemia

OMIM	Disorder	Clinical phenotype	Ref	Gene
145000	Hyperparathyroidism 1	This is the most common familial form of primary hyperparathyroidism. Primary chief cell hyperplasia is the main characteristic of this disorder. Many patients show hypercalcemia.	[134-136]	<i>MEN1</i> <i>HRPT2</i> <i>HRPT3</i>
145001	Hyperparathyroidism 2	Families with this disorder have parathyroid adenomas many of them complicated by fibrous maxillary or mandibular tumors that resemble ossifying fibromas rather than the brown tumors generally found in patients with hyperparathyroidism.	[137] [138] [139] [140]	<i>HRPT2</i>
239200	Hyperparathyroidism, neonatal severe primary	It is thought that this disorder is the result of homozygosity for the gene that in heterozygosity results in familial hypocalciuric hypercalcemia or is due to a recessive mutation at the same locus. It was demonstrated that	[141-143]	<i>CASR</i>

		these disorders are due to mutations in the parathyroid Ca(2+)-sensing receptor. Neonatal hyperparathyroidism is associated with parathyroid hyperplasia but never with parathyroid adenoma. Early diagnosis and parathyroidectomy is necessary to permit survival.		
145980	Hypocalciuric Hypercalcemia, familial, Type I	This was the first disorder described where renal tubular defect in calcium reabsorption is independent of parathormone. A ratio of renal calcium clearance to creatinine clearance below 0.01 suggests FHH1. The only complications attributable to the hypercalcemia are pancreatitis and chondrocalcinosis. Both the kidneys and the parathyroid glands seem insensitive to chronic hypercalcemia.	[144] [145]	<i>CASR</i>
145981	Hypocalciuric Hypercalcemia, familial, Type 2	One different genetic location was identified in a pedigree with familial hypocalciuric hypercalcemia.	[146]	Unknown
600740	Hypocalciuric Hypercalcemia, Familial, Type 3	This form, known as Oklahoma type, represents a different genetic entity. Serum alkaline phosphatase activity and creatinine levels were normal in these individuals, but inorganic phosphate levels were lower than in unaffected members of the kindred. Radiographic studies showed changes suggestive of osteomalacia. Biopsy of the iliac crest confirmed the presence of defective skeletal mineralization.	[147] [148] [149]	Unknown
19405	Williams-Beuren Syndrome Hypercalcemia, infantile	Autosomal dominant disorder with preponderant aspects: supraaortic stenosis and infantile hypercalcemia. Calcification of the aorta and pulmonary artery was reported. Other reports include nephrocalcinosis and microcalcifications in the brain and kidneys. Progressive joint limitation, joint contractures, and scoliosis have been also observed in Williams-Beuren syndrome.	2 [150] [151] [152]	Deletion of several genes on chr7 including <i>ELN</i> , <i>LIMK1</i> and <i>RFC2</i>

Table 7. Disorders characterized by ectopic ossification

OMIM	Disorder	Clinical phenotype	Ref	Gene
135100	Fibrodysplasia Ossificans	Autosomal dominant disorder, rare and characterized by progressive ectopic ossification	[153] [154]	<i>ACVR1</i>

	Progressiva; FOP	of soft tissues, especially after trauma. Phenotypic severity is variable ranging from disabling ectopic bone formation and premature death to asymptomatic adult with characteristic big toe malformations (monophalanic).		
166350	Osseous Heteroplasia, progressive Ectopic ossification, familial POH	Rare autosomal dominant disorder, paternally inherited, characterized by dermal ossification from early infancy, followed by increasing and extensive bone formation in deep muscle and fascia. These patients do not have the characteristic monophalanic great toe seen in FOP. POH is also distinct from tumoral calcinosis.	[155] [156] [157] [158] [159]	<i>GNAS1</i>
103580	Albright hereditary osteodystrophy AHO	This is a syndrome phenotypically characterised by short stature, obesity, round facies, subcutaneous ossifications, brachydactyly and some other skeletal abnormalities. Closely associated with pseudohypoparathyroidism, hypocalcemia and high PTH levels.	[121] [160] [161]	<i>GNAS1</i>
174800	McCune-Albright syndrome MAS	Clinically heterogeneous disorder that normally includes polyostotic fibrous dysplasia (POFD), café-au-lait skin pigmentation and peripheral precocious puberty. It can include various other endocrinologic anomalies such as thyrotoxicosis, pituitary gigantism and Cushing syndrome.	[162] [163] [164]	<i>GNAS1</i>
602475	Ossification of the Posterior Longitudinal Ligament of Spine; OPLL	OPLL is a disorder with familial aggregation considered to be common among Japanese and throughout Asia. It is the result of an abnormal ossification of the spinal ligaments and is the leading cause of myelopathy. Several SNPs in genes such as <i>COL6A1</i> , <i>ENPP1</i> and <i>COL11A2</i> have been found in association with OPLL. A naturally occurring mouse model of this disorder (tiptoe walking mouse) exhibits ossification of the spinal ligaments very similar to human OPLL. The disease causing mutation was identified in the <i>Npps</i> gene (<i>ENPP1</i>). Recent studies found an association between an intronic SNP in the <i>TGFB3</i> gene and OPLL.	[86] [165] [166] [167]	<i>ENPP1</i> <i>COL11A2</i> <i>COL6A1</i> <i>TGFB3</i>
106300	Spondyloarthropathy Marie-Strumpell spondylitis Becherew Syndrome Ankylosing Spondylitis	Spondyloarthropathy is one of the most common chronic rheumatic diseases and includes a spectrum of related disorders comprising the prototype ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis (ReA), arthritis related to inflammatory bowel disease and undifferentiated spondyloarthropathy.	[168, 169]	<i>HLA-B*27</i> Many others
106400	Ankylosing Vertebral Hyperostosis with Tylosis	Diffuse idiopathic skeletal hyperostosis (DISH, Ankylosing Hyperostosis, Forestier-Rotes Querol's disease) is a common condition	[170] [171] [172]	Unknown

Diffuse Idiopathic skeletal hyperostosis (DISH) included	amongst the elderly characterized by ossification of the anterior longitudinal ligament and peripheral entheses affecting, in particular, the right side of the spine with preservation of the intervertebral disc space. Several familial cases of DISH have been described in the literature.
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1.4.3 Idiopathic calcification

Idiopathic ectopic mineralizations occur in tissues for unknown reasons. Diffuse Idiopathic Skeletal Hyperostosis for example, is a disorder characterised by peripheral enthesopathy and by extensive new bone formation within the anterior ligaments of the vertebral column [174]. No cause was yet identified although several metabolic associations have been identified [175].

1.5 MAPPING GENES IN HUMAN DISEASES

1.5.1 Main genetic approach

The process of identifying the genetic basis of a disease is complex and long. It normally follows a certain number of phases [176]:

1. Descriptive epidemiology and generation of hypothesis

In this first step of the process evidences on the importance of genetics and environmental factors should be investigated. A number of studies can be performed to establish if genetic or environmental factors are involved in the etiology of the disease: international variation in disease risk and risk among migrants, racial origin, ethnic backgrounds, socioeconomic class, age and gender differences.

2. Familial aggregation

This step involves looking for evidences of familial clustering. Demonstrating that the disease tends to run in the families more than would be expected by chance will suggest that genetic factors are involved in the etiology.

3. Segregation Analysis

Analyzing the pattern of affected individuals within the families will help to determine if this disease is caused by one or more genes and if there are shared environmental factors involved. If major genes seem to be involved, a genetic model can be suggested.

4. Linkage analysis

Blood samples are collected and informative markers are types on multiples familial cases. Markers that co-segregate with the disease will provide evidences of linked chromosomal regions where the causal gene lie. Linkage analysis is explained in detail later in this chapter.

5. Fine Mapping

Narrowing down the linked chromosomal region is possible by using a dense panel of markers over the candidate region to look for associations that could reflect linkage disequilibrium with the causal gene.

6. Association with candidate genes

The linked region may include many genes that can be relevant to the disease etiology. Comparison of genotypes at the loci of interest between cases and controls can bring to light any association with the disease.

7. Cloning and identification of mutations

When candidate region is satisfactorily narrow and no candidate gene has been found in that region, DNA from that region has to be searched for polymorphisms with various techniques.

8. Characterizing the gene

When the gene is finally identified a series of features are studied using molecular methods. Structure, function and regulatory elements are particularly important. Estimation of frequencies of the various mutations and the effect of each on the disease risk is then exhaustively studied. Interactions with environmental factors are estimated.

Due to its importance in this study, a more detailed explanation of linkage and association analysis is going to follow.

1.5.2 Linkage analysis

Linkage analysis is a valuable tool on genetic epidemiology. It is usually the first stage in the genetic investigation of a trait, since it can identify regions in the genome that contains genes that predispose to disease, by making use of observations of related individuals [177].

This type of strategy relies on the assessment of the recombination information from the different generations available in the investigated families.

Recombination fraction defines genetic distance between two loci. Where there is little or no apparent recombination between a marker and the putative disease-causing gene, the marker is said to be linked with the disease. So, as it was previously mentioned, the goal of linkage strategy is to know how often two loci are separated by meiotic recombination. If two loci are on different chromosomes they will segregate independently. Thus, on average, 50% of the offspring will be recombinant (recombination fraction = 0.5). If the loci are syntenic (lie on the same chromosome) then they might always segregate together with no recombinants. However, this simple rule is ignoring meiotic crossovers. A crossover, if it occurs between two loci, will create two recombinant chromatids (recombination fraction = 0.5 between loci flanking it). Recombination will rarely separate loci that lie very close together on a chromosome. Therefore, sets of alleles on the same small chromosomal segment tend to be transmitted as a block through a pedigree. This block of alleles - haplotype - can be tracked through pedigrees and through populations and, when not broken up by recombination, haplotypes can be treated for mapping purposes as alleles at a single highly polymorphic locus.

Parametric analysis

Parametric analysis is a model based linkage analysis that requires precise genetic information, detailing the mode of inheritance, gene frequencies and penetrance of each genotype. Evidence for linkage is normally expressed as logarithm of the odds score - LOD score.

LOD score

In large families it is not always possible to identify recombinants. However, it is possible to calculate the likelihood of the pedigree, on the alternative assumptions that the loci are linked (recombination fraction = θ) or not linked (recombination fraction = 0.5). The ratio of these two likelihoods gives the odds of linkage, and logarithm of the odds is the **lod score**. It has been demonstrated that lod scores represent the most efficient statistical way of evaluating pedigrees for linkage. Positive lod scores will give evidence in favour of linkage, while negative lod scores will give evidence against linkage. For whole genome studies a genome wide threshold of significance must be used. There has been much

debate over this subject. In theory, the appropriate threshold for significance is a lod score such that there is only a 0.05 chance of a false positive result occurring anywhere during a search of the whole genome. A lod score of 3.0 corresponds to a significance of 0.05 at a single point. However, the chance of a spurious positive result increases with the number of markers used in the study. A rigorous procedure consists in multiplying the p value by the number of the markers used before testing its significance - Bonferroni correction. Thus, the threshold lod score for a study using n markers would be $3 + \log(n)$, that is 4 for 10 markers, 5 for 100 markers, and so on. However, and because linkage data are not independent, this is over stringent. It is widely accepted that, for Mendelian characters, the threshold for a genome-wide significance level of 0.05 is 3.3. In practice, lod scores below 5 should be regarded as provisional [178].

To increase the efficiency of linkage analysis the loci should be analyzed simultaneously. Multipoint mapping is more efficient than two-point mapping. It is also the best way to overcome problems caused by limited informativeness of markers. This is less important with microsatellite markers but highly important with RFLPs and SNPs [178].

If the genetic component of the disease in study is made up of a large number of distinct genes, parametric linkage analysis can be severely compromised and model-free alternatives need to be used.

Non-parametric analysis

For multifactorial diseases in which several genes, as well as environmental factors, contribute to disease risk, there is no clear mode of inheritance. Methods that are non-parametric or model-free have been developed to investigate linkage in this type of diseases. Assuming that affected relatives share haplotypes that are identical by descent (IBD) in the region where lies the disease-causing genes, the methods will test if the IBD sharing at a locus is greater than expected under the null hypothesis of no linkage.

Sibling Pairs

The straightforward approach to non-parametric linkage is to investigate affected siblings. At any locus, according to the hypothesis of no linkage, the number of

IBD alleles shared by a pair of siblings is none or two, with a probability of 0.25, and one with a probability of 0.5. If IBD sharing is known, linkage would be suggested if the affected siblings share significantly more alleles than expected by chance.

Relatives groups

Alternative methods, based on the degree of IBD sharing, have been developed to analyze larger numbers of individuals, of differing relationship, within the studied family. The score, that measures IBD sharing, can be based in pairwise comparisons but another alternative score was proposed [179], that increases as the number of affected pairs sharing the same IBD allele increases. To solve problems, related to large size pedigrees and use of large number of markers, methods have been developed based on Markov chain Monte Carlo estimation [180].

1.5.3 Association studies

This type of strategy aims to detect association, in affected and unaffected individuals, between one (or more) genetic marker and a trait, based on the assumption that human populations share common ancestry.

Association differs from linkage in that the same allele is associated to the trait in a similar manner across the whole population, while linkage allows different alleles to be associated with the trait family wise [181].

Association studies have greater power than linkage studies to detect small effects, but requires the use of many more markers. Special attention to population stratification or admixture is required in study design.

Study designs for genetic association studies include:

Cross-sectional

Cross-sectional studies make use of a random sample from population in which genotypes and phenotypes are studied. One advantage of this study is that it provides an estimate of disease prevalence in that population. Another advantage is the inexpensiveness of the process. A big disadvantage is that it cannot be

used when dealing with rare diseases in which only a few affected individuals exist in the populations. Statistical methods that can be used in the evaluation of the results include logistic regression, Chi-square tests of association or linear regression.

Cohort

Cohort studies involve, usually, the genotype subsection of population and follow the disease incidence for a specified time period. It thus provides an estimate of disease incidence. Major disadvantage of this type of strategy: it is expensive to follow-up and there are always drop-outs that may affect the results. Statistical analysis methods used for the analysis of these results are the so called survival analysis methods.

Case control

Case control studies are a classic type of association studies. They aim to compare allele frequencies or genotypes between a sample of unrelated affected individuals and a sample of matched controls from population or convenience sample. The advantages of this strategy are: it is not necessary to follow-up the individuals; it is not necessary to spend time trying to know the family history; it provides an estimate of exposure effects. Disadvantages are the requirement of careful selection of controls and the potential for confounding (e.g. population stratification). Results are evaluated through logistic regression or chi-square tests of association.

Family based designs (Case-parent triads / Case-parent-grandparent septets / general pedigrees)

Affected cases are genotyped plus their parents and grandparents. The major advantage of this type of study is the robustness to population stratification. It is also possible to evaluate maternal and paternal imprinting effects. This strategy is less powerful than case-control design and requires the participation of family individuals, which is not always possible. Statistical analysis methods include transmission disequilibrium tests (TDT), logistic regression or log-linear models.

Extreme Values

This type of study utilizes only the individuals with extreme values (high or low) of a quantitative trait. Only these are chosen for genotyping. The advantage of this type of study is that costs are lower due to the smaller number of individuals. However, there is no estimation of the real genetic effect. The statistical analysis of the results is performed by linear regression, non-parametric or permutation approaches.

Case-only

This is a powerful method to detect interaction effects. Only affected individuals, obtained from a cross-sectional, cohort or disease-outcome based sample, are genotyped. Major disadvantage of this strategy is that it is very sensitive to population stratification. Logistic regression chi-square tests of association are used to evaluate the results.

1.6 EPIDEMIOLOGY AND PATHOGENESIS OF DISH AND CPPD-CC

1.6.1 DISH

Diffuse Idiopathic Skeletal Hyperostosis [DISH; MIM 106400] is the current terminology for a disorder first reported in 1925 by Knaggs [182].

This disorder has been known under a variety of names in international literature. The terminology of this disorder has been successively changed by researchers, due to the main characteristics of phenotypes encountered in the investigated groups. Main designations for DISH can be seen in table 9.

Table 9. Former and present designations for DISH

DISH designation	Authors	Year	Ref
Spondylitis deformans	Knaggs, R.L.	1925	[182]
Spondylitis ossificans ligamentosa	Oppenheimer, A	1942	[183]
Senile ankylosing hyperostosis of the spine	Forestier and Rotes-Querol	1950	[184]
Vertebral osteophytosis	Lacapierre, L	1951	[185]
Spondylosis hyperostotica	Ott, V.R.	1953	[186]
Physiological vertebral ligamentous calcification	Smith et al.	1955	[187]
Generalized juxtaarticular ossification of vertebral ligaments	Sutro et al.	1956	[188]
Ankylosing hyperostosis of the spine	Forestier and Lagier	1971	[189]
Diffuse Idiopathic Skeletal Hyperostosis	Resnick et al.	1975	[190]

Diffuse idiopathic skeletal hyperostosis is a common condition amongst the elderly characterized by ossification of the anterior longitudinal ligament (ALL) affecting, in particular, the right side of the spine with preservation of the intervertebral disc space. Whilst spinal involvement in DISH is nearly universal, in some cases peripheral entheses may also be involved, where ossification and bony spurs are seen [172, 191].

1.6.1.1 DISH diagnostic criteria

There are two main diagnostic criteria sets to identify definite, probable or possible patients affected with DISH. Resnick defined the first set of criteria that were, some years later, revised by Utsinger for epidemiological purposes (table 10).

Table 10. DISH diagnosis criteria established by Resnick and Utsinger

		Resnick Criteria [81]	Utsinger Criteria [172]
Definite	1	<i>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.</i>	<i>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.</i>
	2	<i>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.</i>	<i>Continuous ossification along the anterolateral aspect of at least two contiguous vertebral bodies.</i>
Probable	Possible 3	<i>The absence of apophyseal joint bony ankylosis and sacroiliac joint erosion, sclerosis or intraarticular osseous fusion.</i>	<i>Symmetrical and peripheral enthesopathy involving the posterior heel, superior patella or olecranon, with the enthesal new bone having a well-defined cortical margin.</i>

According to the criteria previously presented, categories of DISH are as follows: Definite: 1, Probable: 2, 3, Possible: 2 and 3, 2, 3 (particularly if calcaneal spurs occur together with olecranon or patella spurs).

DISH diagnosis exclusions are made if:

1. There is abnormal disc space height in the involved areas.
2. There is apophyseal joint ankylosis.

Resnick criteria number 3 had an exclusion factor based on the erosions, sclerosis or fusion of sacroiliac joints. This would help excluding patients with ankylosing spondylitis. This exclusion factor was withdrawn by Utsinger because

differentiation of these two disorders should be possible with lateral and antero posterior axial x-rays. Furthermore, the co-existence of DISH and ankylosing spondylitis is possible and has been thoroughly described in more than 10 reports in recent years [192-205]. Co-existence of DISH with other disorders have been related since the first report of this disorder by Forrester and Rotes Querol, in 1950 [184]. It has been reported that 50% of DISH cases are complicated by a similar condition, ossification of the posterior lateral ligament (OPLL, MIM 602475) [206] suggesting that they share common aetiopathogenic factors. OPLL is mostly found in Asian populations, in particular amongst Japanese, where it has been reported to have a prevalence of 1.9 to 4.3% [206-212]. Other rheumatic diseases found co-existing with DISH include: hyperostosis frontalis interna [211, 213-216], chondrocalcinosis [173, 217], gout [217-220], rheumatoid arthritis [217, 221-223], Heberdens and Bouchard nodes [224], osteoarthritis [217], and Paget's disease [214, 225]. It is often found the association of DISH with psoriatic arthritis in the literature. Studies concluded that it is the result of treatment with retinoids that causes enthesopathic problems [226]. Studies have reported other metabolic unbalances that can lead to enthesopathic ossification: ochronosis, hypophosphatasia, acromegaly, haemochromatosis, hypomagnesaemia, fluorosis, and hypervitaminosis A, may also cause similar enthesopathy [227].

1.6.1.2 DISH prevalence

There have been only a few large studies on DISH prevalence. The first large study was performed in two large American Midwest Metropolitan Hospital Population groups. This group found that the overall prevalence of DISH in the male population over age 50 years was 25% and in the female population over age 50 years was 15%. This prevalence rose to 28% in males over 80 years and to over 35% in males over age 70 years. In females over 80 years, the prevalence was found to be 26%. The overall prevalence of DISH was higher than expected in a predominantly white population over age 50 years with a lesser incidence in the black, Native-American and Asian populations, suggesting a genetic origin of the condition [228]. This study corroborates a previous study performed on a considerably smaller group constituted of African blacks that stated that the overall prevalence of DISH was 3.9% (males 3.8% and females 4.2%). The authors

noted a rise in the prevalence of DISH with increasing age from 1% in the 40-49 year age group to 13.6% in those over 70 years. They reported a high prevalence of diabetes (52.4%) in the 21 patients with DISH who were seen in the prospective analysis [229].

One of the latest prevalence reports was performed in Korea in a quite large Asian population group. The authors confirmed one other study [230] which suggested that the overall DISH prevalence is much lower in Koreans than in other Western populations. The authors suggest that ethnic factors are important elements in the prevalence of DISH [231]. The results of these studies are somewhat surprising since OPLL, a disorder very similar and frequently seen in association with DISH, is highly prevalent in Asian populations. One would normally expect a high prevalence of DISH in this population. Further studies should be performed to enlighten this subject. This prevalence diversity may be very helpful in the understanding of DISH etiology.

The most recent large prevalence study was performed on an outpatient population in the Netherlands where the authors came to the conclusion that the prevalence of this disorder was higher than the previously reported. The overall prevalence of DISH was 17.0% (95% CI 13.7-20.3). They have observed a significant increase with age (odds ratio 1.03, 95% CI 1.01-1.05; $p = 0.006$). The odds ratio of male sex was 1.85 (95% CI 1.20-2.86; $p = 0.006$). DISH prevalence was 32.1% in 80-year-old men and 16.9% in women of the same age. Early radiographic changes of DISH were found in 4.6% of the individuals and were more frequent in women.

Besides the prevalence of DISH in this cohort (17%) the authors concluded, similarly to other studies, that age and sex were significantly related to the presence of DISH, leading to the suggestion that men and older individuals have a higher probability of developing DISH [232].

1.6.1.3 DISH aetiopathogenesis

The aetiopathogenesis of DISH remains unknown; however, its frequent association with several metabolic conditions may give some clues concerning its aetiopathogenesis. Some biochemical associations with DISH include a higher

insulin and growth hormone levels, possibly explaining the osteoblast cell growth and proliferation [233], a greater body mass index (BMI), a higher serum level of uric acid and a higher likelihood of having diabetes mellitus [234]. The association of DISH with diabetes mellitus has been much investigated in recent years [235, 236]. It is believed that the consequence of the previously mentioned biochemical facts in the etiopathogenesis of DISH is the abnormal cell growth/activity in the bony – ligamentous region [237, 238]. It is known that insulin like growth factor (IGF)-I stimulates alkaline phosphatase activity and type II collagen in osteoblasts and that growth hormone can induce the local production of IGF-I and IGF binding proteins in chondrocytes and osteoblasts.

It is believed that Matrix Gla Protein (MGP) action includes the inhibition of mineralization by suppression of bone morphogenetic protein 2 (BMP-2) which is a potent osteogenic factor. Sarzi-Puttini et al reported higher serum MGP concentration levels in male and female DISH patients, when compared with healthy controls. This group suggests that MGP may be a marker of hyperostosis because it is produced in larger amounts by patients with hyperostosis – inducing osteometabolic syndromes such as DISH [239]. MGP is an 84 residue vitamin K dependent protein expressed in various tissues and upregulated in bone cells. The MGP gene spans 3.9kb and consists of 4 exons separated by 3 large intervening sequences which account for more than 80% of the gene [240].

1.6.1.4 DISH genetics

DISH is frequently seen in association with another paravertebral ligamentous ossification; OPLL. Some studies have found that OPLL is seen in up to 50% of the patients with DISH [206, 241, 242]. 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 [167].

There have only been a few genetic studies on DISH patients most of them based on its association with OPLL.

There have been many studies investigating the possible involvement of Human Leukocyte Antigen (HLA) alleles with OPLL [233]. Koga and colleagues performed

a genetic linkage study in 91 OPLL affected sib pairs and report a predisposing locus for OPLL, on chromosome 6, close to the HLA complex. After analysis of genetic variants it was suggested that the candidate gene *COL11A2* may be associated with the disease [243]. Maeda et al continued to investigate the association of *COL11A2* with OPLL and report an association of a gender-specific *COL11A2* haplotype with the disease. However, this association could only be seen in male patients [244].

To elucidate the genetics basis of OPLL Okawa et al investigated the *ttw* (tiptoe walking) mouse, a naturally occurring mutant which exhibits similar anomalies in the spinal ligaments to the OPLL patients [86]. Using a positional candidate-gene approach this group identified a nonsense mutation (Glycine 568 to a Stop Codon) in the *Npps* gene as the causation of *ttw* phenotype. The homologous human gene is *ENPP1*. As described above it encodes an enzyme that is responsible for the production of inorganic pyrophosphate, a major inhibitor of calcification, thus regulating soft tissue calcification and bone mineralization [165].

In 2001, novel mouse genes associated with ectopic ossification were isolated by differential display method using the same mouse model – *ttw*. This mouse is an excellent model for ectopic ossification because it exhibits an ossification pattern histologically similar to human OPLL. To clarify the molecular mechanism of ectopic ossification in *ttw*, the authors examined the effect of dietary phosphate and calcium on the *ttw* phenotype and found a high dietary phosphate-accelerated ectopic ossification. Genes associated with the enhanced ossification on a high phosphate diet were identified by a differential display method. Nine mouse genes were identified; six were up-regulated by the high phosphate diet, and three were down-regulated. Six of the nine genes were novel and were cloned and characterized. Two of the genes were highly specific to cartilage, suggesting their specific role in enchondral ossification [245]. This novel identification should have given novel insights into the mechanism of ectopic ossification and etiology of OPLL. However, the exact genetic cause for OPLL is still unknown.

The association of *TGFB1* with OPLL has been postulated to be related to its role in the growth and differentiation of cartilage, enchondral and membrane bone and skin. Some studies have shown significant associations between variants of the gene and the prevalence of OPLL in the cervical spine [246]. A later study

examined the relation between the previously associated polymorphism (T869>G) and the radiologic characteristics of OPLL. This study concluded that this polymorphism is not a factor associated with the occurrence of OPLL but rather a factor related to the area of the ossified lesion [247].

To identify new genetic loci related to OPLL Tanaka and colleagues performed a genomewide linkage study with 142 affected sib-pairs. After multipoint linkage analysis, evidence of linkage to OPLL was detected in 6 zones of different chromosomes. The best evidence of linkage was found on a zone of chromosome 21 that was extensively investigated, with single nucleotide polymorphisms (SNPs), for linkage and association studies. After case-control comparison, *COL6A1*, lying precisely on chr.21 (21q22.3), was the gene most significantly associated with OPLL [166].

A large-scale study investigating the genetics of OPLL was performed with the objective of identifying new susceptibility genes for OPLL and to re-examine previously reported associations. Thirty five candidate genes were genotyped in sporadic OPLL patients (n=711) and controls (n=896). Significant associations were found in three genes; the most significant association was found in *TGFB3* gene (P=0,0004). Previously reported associations of *COL11A2*, *ENPP1* and *TGFB1* were not reproduced in this study [248].

1.6.2 CPPD chondrocalcinosis

Chondrocalcinosis, which may have many different causes, can be considered as a specific form of ectopic calcification [4]. It is a disorder characterised by the deposition of calcium salts in articular, including meniscal, cartilage. The deposited salts are normally composed of calcium pyrophosphate dehydrate (CPPD), though other calcium salts can also be found including hydroxyapatite.

1.6.2.1 CPPD chondrocalcinosis diagnostic criteria

Criteria for CPPD CC diagnostic include, at least, two of the following (A or B and C):

- A. Identification of calcium pyrophosphate crystal in tissue or synovial fluid by X-ray diffraction from joint aspiration or biopsy
- B. Identification of absent or weakly positive birefringence crystals from joint aspirate
- C. Presence of typical radiographic calcifications

1.6.2.2 CPPD chondrocalcinosis prevalence

The most recent CC prevalence study was performed with Chinese subjects that showed to have a much lower prevalence of knee CC (1.8% in men, 2.7% in women) when compared with caucasian subjects (6.2% in men, 7.7% in women). The age-standardized prevalence ratio was 0.34 (95% confidence interval [95% CI] 0.20-0.54) and 0.43 (95% CI 0.31-0.59) in men and women, respectively. Wrist CC was rare in elderly Chinese subjects (prevalence 0.3% in men and 1.0% in women), with the age-standardized prevalence ratio being 0.06 (95% CI 0.01-0.18) in Chinese men and 0.18 (95% CI 0.10-0.30) in Chinese women. The authors compare the results obtained in this study with another study performed with white subjects from the Framingham Study (US), concluding that knee and wrist CC are far less common in Chinese [249]. The Framingham Study confirmed the association of Osteoarthritis (OA) with CC, being the relative risk = 1.52 (95% CI 1.22, 1.90), after controlling for age. CC was shown to be positively associated with both symptomatic and asymptomatic radiographic OA. The proportion of radiographic OA potentially attributable to CC was only 4.4%. The results of this study confirm that CC is significantly associated with OA after controlling for age, but they also suggest that chondrocalcinosis and OA increase independently with age [250]. The association of OA with CC has been subject of intense debate. There is a considerable overlap between these two disorders and more detailed studies are necessary to specify the exact liaison between both disorders.

There have been only a few studies to determine the prevalence of CC in first-degree relatives of patients with apparently sporadic CC or pyrophosphate arthropathy. Recurrence risk rates in first-degree relatives of 11% to 38% have been reported [[251],[252],[253],[254]]. However, these studies have several limitations including the small number and mode of selection of index cases, the

elevated non-participation rate and most importantly, the absence of a community control population.

Only one study has been published comparing the frequency of CC in siblings of cases compared with a control population [255]. This study showed an increase in the unadjusted recurrence rate in siblings compared with the general population (OR = 2.01, 95% confidence interval (CI) 1.13 to 3.56), but after adjustment for age, BMI, gender, knee pain and knee OA, this became non-significant. It is unclear as to why the adjustment for knee pain and OA was made, and therefore the familiarity of late-onset chondrocalcinosis remains uncertain. This is not unusual in late-onset diseases, where genetic effects do not easily translate into increased familial recurrence, because of incomplete penetrance. Given the number of monogenic causes of chondrocalcinosis, it is highly unlikely that genetic variation is not going to affect susceptibility to the disease in later onset, 'sporadic' forms.

1.6.2.3 CPPD chondrocalcinosis aetiopathogenesis

Chondrocalcinosis can occur either as a dystrophic calcification in damaged cartilage and in older people or as a metastatic calcification in association with several disorders where there is an acquired or inherited metabolic abnormality such as hyperparathyroidism, haemochromatosis, hypophosphatasia, ochronosis and Wilson's disease, among others (see table 8).

Table 8. Metabolic disorders associated with CC:

OMIM	Disorder	Clinical phenotype	Ref	Gene
145980	Hypocalciuric hypercalcemia, familial, Type I	This was the first disorder described where renal tubular defect in calcium reabsorption is independent of parathormone. A ratio of renal calcium clearance to creatinine clearance below 0.01 suggests FHH1. The only complications attributable to the hypercalcemia are pancreatitis and chondrocalcinosis. Both the kidneys and the parathyroid glands seem insensitive to chronic hypercalcemia in this disease.	[144] [145]	CASR
203500	Alkaptonuria	Autosomal recessive disorder characterised by the deposition of ochronotic pigment in cartilage and connective tissues.	[256]	HGD
248250	Gitelman syndrome	Variant of Barter Syndrome in which patients	[257-	SLC12A3

		present hypokalemic alkalosis in conjunction with hypocalciuria and hypomagnesemia.	259]	
607364	Bartter syndrome Type III	Autosomal recessive heterogeneous disorder characterized by impaired salt reabsorption in the thick ascending loop of Henle with pronounced salt wasting, hypokalemic metabolic alkalosis, and hypercalciuria. CC and nephrocalcinosis are major symptoms of this syndrome.	[260]	CLCNKB
277900	Wilson disease	Autosomal recessive disorder characterized by massive amounts of intracellular hepatic copper causing hepatic and neurologic abnormalities. CC and OA are often seen in these patients.	[261]	ATP7B
235200	Haemochromatosis	Group of phenotypically and genetically heterogeneous disorders characterised by iron overload. CPPD-CC has a well known association with haemochromatosis affecting about 67% of the patients.	[262, 263]	HFE TFR2 HJV HAMP SLC40A1
146300	Hypophosphatasia, adult	Heterogeneous biochemical and clinical features which include: low levels of serum alkaline phosphatase, high levels of serum pyridoxal-5-prime-phosphate, early loss of teeth (odontohypophosphatasia), bowed legs, calcification of paraspinous ligaments, joint pains and peri and intraarticular calcifications of joints of the hands, feet and knees and calcification of the anterior spinous ligament in the lumbar area.	[125] [264] [126] [127] [128]	ALPL

Much has been learnt about the control of mineralization through genetic studies, particularly through identification of genes involved in monogenic forms of disorders of ectopic mineralization [265]. Mutations in several of these genes affect the rate of generation, transport and degradation of PPI and have been quite useful in investigating the mechanisms controlling mineralisation [25]. This has been particularly helpful in characterisation of CPPD deposition diseases, where there are currently no animal models, and in vitro studies are of uncertain relevance to the in vivo situation.

It is known that inorganic pyrophosphate (PPI) antagonizes the formation of HA crystals hence the importance of having a tight balance between the levels of extracellular Pi and PPI for normal mineral deposition [[266],[25]]. Factors controlling the rate and tissue specificity of mineralization have been extensively investigated, and include factors affecting transport and metabolism of PPI,

factors which affect crystal nucleation, and components of the extracellular matrix, such as the presence of fibrillary collagen [267].

As seen in table 8, several monogenic diseases are complicated by CPPD chondrocalcinosis, and in most cases the disease mechanism appears to be through effects on P_{pi} levels. This may be the result of increased levels of intracellular P_{pi} due to decreased TNAP (ALP) activity (high levels of ALP inhibitors such as calcium, copper or iron or low levels of ALP cofactor: magnesium), increased calcium concentrations, enhanced crystal nucleation (iron and copper as in haemochromatosis and Wilson's disease, respectively) or decreased crystal solubility (as in hypomagnesemia) [268].

Probably the most common disease association of CPPD chondrocalcinosis is haemochromatosis, but the mechanism of this association is still poorly understood. Opposite effects of iron salts on CPPD crystal formation have been reported by different investigators, but it is generally thought that they promote crystal nucleation and/or growth [[269],[270]]. It has also been suggested that iron may inhibit pyrophosphatases [271]. It is obvious that much needs to be investigated in this particular field.

Hypophosphataemic rickets is another fascinating condition, in which either ectopic hydroxyapatite or CPPD crystal deposition occurs. Whilst physiological skeletal mineralization is impaired, thought to be due to the low P_i levels, ectopic hydroxyapatite mineralization can be very problematic, causing spinal stenosis and widespread calcific enthesitis. The cause of this is unclear, but may be due to low P_{pi} levels permitting extraskeletal mineralization. If this is the case, then long-term phosphate treatment, even in adulthood, may play a role in preventing this complication of the condition.

1.6.2.4 Chondrocalcinosis genetics

Although most cases of CPDD are non-familial, there are many reports of kindreds with a familial form of this disorder, indicating a significant genetic impact in the disease. Since the first report of a familial form of CPDD by Zitnan and Sitaj [272], designated as "chondrocalcinosis articularis", many other reports of familial aggregation have been reported scattered throughout the world [[273]-[274],[253]]. Two main different clinical phenotypes have been observed in all

these kindreds. The first is characterized by the early onset, polyarticular involvement, and variable severity of arthropathy; the second is similar to that observed in sporadic disease cases and is characterised by late-onset oligoarticular CC with arthritis [275]. All these reports are characterised by the strong heterogeneity of phenotypes within and between families, and in most cases, by autosomal dominant transmission of disease.

1.6.2.4.1 Monogenic forms of chondrocalcinosis

CPPD-CC segregating as an autosomal dominant trait has been described in many families. Two chromosomal regions have been linked to this disease. In 1995, Baldwin and his colleagues [276], reported genetic linkage between markers on human chromosome 8q and the disease in a large family from Maine with early onset CPPD and severe degenerative osteoarthritis (OA) [CCAL1, MIM 600668]. The disease causing gene has yet to be mapped but there is a strong possibility that this gene is primarily related with osteoarthritis and that the CPPD deposition is secondary, enhanced by the degenerative changes in cartilage.

ANKH

Greater success has been experienced with a second form of autosomal dominant CPPD deposition disease CCAL2 [CCAL2, MIM 118600]. This condition was initially described and genetically localised in five English families with CPPD crystal deposition. Linkage was established to chromosome 5p, with a multipoint LOD score of 4.6 [277]. Subsequently, in two CPPD-CDD unrelated families, one from Argentina and the other from France, another zone in chromosome 5p, mapping just centromeric to the first, was further identified through recombination analysis [278]. The identification of the *ank* gene, and demonstration that loss-of-function mutations in the gene cause ectopic hydroxyapatite deposition in the *ank* mouse model [31], led investigators to postulate that gain-of-function mutations in the human homologue of this gene may be responsible for the disease in these families [268]. This finding has subsequently been confirmed in other families with autosomal dominant CPPD deposition disease [279] [32, 280].

Mutations in *ANKH* have also been shown to cause another autosomal dominant condition characterised by hyperostosis and sclerosis of the skull, together with flaring and abnormal modelling of the metaphyses of long bones – Jackson’s craniometaphyseal dysplasia [CMD, MIM 123000] [281]; [85]. As discussed below, this disease has many similarities with the ank mouse model.

Despite knowing that *ANKH* mutations are the cause of skeletal problems, until recently, very little was known about how these mutations affect the protein function. To elucidate this subject, the role of ANK in arthritis and bone disease was investigated in a series of experiments performed by Gurley et al. [282]. Wild type and mutant forms of ANK associated with CC (P5T and M48T) and CMD (C331R and G389R), were tested *in vitro* for radiolabeled PPi-transport activity in frog oocytes. Two human mutations, M48T (CC associated) and G389R (CMD associated) were also tested *in vivo*, reconstructed in a bacterial artificial chromosome and tested in transgenic mice for rescue of the ANK null phenotype and induction of new skeletal phenotypes. It was finally made clear that ANK has a role of physiological relevance by actually transporting PPi across the plasma membrane. However, this study does not exclude the possibility of ANK indirect regulation of a separate PPi transporter.

Previous studies investigating the result of *ANK* mutations relied on measurements of bulk PPi inside the cells and in the milieu surrounding them, after some days of culture. This method can give rise to significant errors due to the numerous sources of PPi generated as a by-product of metabolic reactions. In this study both tested mutations (P5T and M48T) retain substantial PPi transport activity, a rate that is similar to that of the wild-type protein. It was never demonstrated that the mutant protein has an activity higher than the wild type protein. However, the hypothesis of a gain-of-function of ANK protein is still the most credible since patients with CCAL2 present CPPD crystal deposition. Tests *in vivo* confirmed that the transgenic M48T-mutant ANK protein was capable to rescue the joint defects of Ank null mice. However, there was a total absence of CCAL2 phenotypes in transgenic mice.

A recent study reported the first family with the *ANKH* mutation G389A in which CMD and CC co-segregated in some affected family members [283]. Affected members of this family presented unilateral and bilateral facial nerve palsy, progressive hearing loss and episodic severe joint pain in hands, feet, shoulder and elbows. One affected individual had calcium deposits in shoulder and elbow

joints. Synovial fluids analysis revealed the presence of calcium pyrophosphate crystals. Interestingly, not all the individuals who had the mutation had CMD or CC phenotypes.

Another study investigated the role of wild-type Ank and its mutants P5L, P5T, M48T, in the regulation of P_{PPi} transport *in vitro*, using ATDC5 cells [284]. The activity of two important pyrophosphate-modulating enzymes, nucleotide pyrophosphohydrolase phosphodiesterase (NPP) and alkaline phosphatase was also evaluated. These authors observed that only the P5L mutant resulted in increased activity of NPP and resulted in increased elaboration of extracellular P_{PPi} at the proliferative and nonmineralizing hypertrophic stages of differentiation. This effect is not observed in the other mutations tested. It is the author's opinion that the interaction between ANK and ENPP1 is interesting and deserves further investigations.

The exact mechanism by which *ANKH* mutations cause these diseases is unknown. Part of the difficulty in determining the function of ANK and effects of *ANKH* mutations may relate to the technically challenging nature of measurement of extra- and intra-cellular P_{PPi}, and the fact that available chondrocyte cell lines are not very close matches to primary cells. The absence of a murine model of CPPD-CC is a particular weakness. The development of such a mouse model would greatly advance our ability to investigate the aetiopathogenesis of this disease.

Several other roles for *ANKH* have been suggested by genetic studies. Besides cartilage, Ank is widely expressed in the brain, particularly in the thalamus, midbrain, and spinal cord, and its expression is influenced by seizure activity. One family with autosomal dominant CPPD-CC known to be due to *ANKH* mutations is also characterised by a seizure disorder, and the gene has also been investigated as being a possible candidate for idiopathic seizure disorders [285]. *ANKH* polymorphisms have been reported to influence a variety of skeletal size parameters [286] and osteoprotegerin levels [287], but these findings are not robust and await confirmation.

The association of *ANKH* with Ankylosing Spondylitis (AS) is also rather controversial. The *ank* mouse initially came to attention as a potential model of human AS. Further, reports of a clinical association between AS and another disorder of ectopic hydroxyapatite deposition, OPLL, raised the possibility that

ANKH may play a role in the pathogenesis of spinal ossification in AS. While a study performed by Tsui et al. [288] reported a modest association of *ANKH* to AS susceptibility, a study performed by Timms et al. [289] showed no evidence of association of *ANKH* polymorphisms with either susceptibility to or severity of disease, nor was any association observed in the Wellcome Trust Centre Case-Control Consortium (WTCCC) SNP study [290]. Additional studies will be necessary to elucidate this subject.

1.6.2.4.2 Other candidate-genes in chondrocalcinosis

ENPP1

There is genetic evidence suggesting the involvement of the enzyme ectonucleotide pyrophosphatase 1 (ENPP1) in ectopic calcification disorders such as the OPLL [291] and arterial calcification [87]. The normal activity of this enzyme, a membrane bound glycoprotein which produces inorganic pyrophosphate from ATP, inhibits pyrophosphate related calcification and mineralization. The *ENPP1* gene maps to chromosome 6 (6q22-q23) and comprise 25 exons that encode a protein expressed in a variety of tissues including matrix vesicles of bone and cartilage. The mouse model of OPLL, “tiptoe walking” (*ttw*) mouse, results from a naturally occurring nonsense mutation in the gene encoding ENPP1 (*Enpp1*^{-/-}). It exhibits ossification of the spinal ligaments and articular cartilage calcification by hydroxyapatite deposition [86]. Mutations in *ENPP1* cause the condition idiopathic infantile arterial calcification, a condition characterised by periarticular and arterial hydroxyapatite deposition, with low activity of ENPP1 and low PPI levels [87]. It is thus possible that gain-of-function mutations in this gene are involved in the development of CPDD, though preliminary studies do not identify a major effect on CPPD risk from common genetic variants of *ENPP1* [292].

TNAP

Tissue-Nonspecific Alkaline Phosphatase (TNAP) is an isozyme of a family of four homologous human alkaline phosphatase genes. It is present in the matrix vesicles and has the ability of hydrolyze PPI. In humans, this enzyme is encoded by the gene *TNAP*, that maps in chromosome 1, containing 12 exons.

Deactivating mutations in the *TNAP* gene cause hypophosphatasia [MIM 241500 (Infantile form), MIM 146300 (Adult type)], characterized by poorly mineralized cartilage and bones, spontaneous bone fractures, CPPD-CC, and elevated concentrations of PPI [293].

Using double knockout mice, lacking both *TNAP* and *ENPP1* genes, Hessle et al have shown evidences that *TNAP* and *ENPP1* are key regulators of bone mineralization suggesting that these genes are antagonistic regulators in this process [294].

To evaluate the hypothetical role of *ENPP1* and *TNAP* in CPDD, Zhang et al [292] screened variants of *ENPP1* and *TNAP* in 128 sporadic, severe, early onset Caucasian CPPD-CC patients and 600 healthy controls. No significant difference was observed in the alleles or genotype frequencies between patients and controls suggesting that these gene are not major genetic determinants of sporadic CPPD susceptibility in white Caucasians [292].

OPN

A recent study performed by Rosenthal *et al.*, highlighted the importance of osteopontin (OPN) as a modulator of CPPD crystal formation in articular cartilage, using a well-characterised, chondrocyte-based model. This study has shown that osteopontin is present in the extracellular matrix near CPPD crystal deposits in articular cartilage and that it stimulates CPPD crystal formation by articular chondrocytes *in vitro* [295]

CILP/NTPPH

Cartilage intermediated layer protein/nucleotide pyrophosphohydrolase (CILP/NTPPH) has been investigated because it was previously observed that its specific activity in the ultrasedimentable fraction of joint fluids from patients with CPPD crystal deposition disease is higher than that in joint fluids from normal individuals and patients with OA, rheumatoid arthritis (RA) and bursitis [296]. This gene was then excluded, by analysis of a radiation hybrid panel, as the cause for certain familial forms of CPPD-CDD because it maps to chromosome 15q21 [297]. However, another study has demonstrated that the fluctuation of CILP/NTPPH expression with age and in response to growth factors parallels changes in ePPI elaboration. These findings suggest that increased CILP/NTPPH

expression with age might promote CPPD crystal formation in aged cartilage [298].

1.6.3 Co-existence of CPPD-CC and DISH

An investigation performed by Bruges Armas *et al* in the Azores, Portugal, identified a series of families characterised by both CPPD-CC, and florid ligamentous calcification, consistent with the condition diffuse idiopathic skeletal hyperostosis [DISH; MIM 106400][274]. The identification of CPPD crystals in all synovial fluids examined suggests that these families have a form of autosomal dominant CPPD deposition disease; however unlike previous reports of this condition, in these families, the CC was in all cases complicated by peripheral and axial enthesopathic calcifications. Linkage to chromosomes 5p15 (CCAL2) and chromosome 8p (CCAL1) was excluded by microsatellite marker studies. Further genetic studies were performed and others are currently ongoing. It is well known that CPPD-CDD has been considered as one of the great mimickers in Rheumatology. It remains to be seen if whether this is a new disease or, on the contrary, an unusual familial variant of DISH or chondrocalcinosis. The identification of these families was the starting point for this thesis. This association of disorders or, perhaps, this endemic disorder, will be addressed in detail later in this thesis.

2. AIMS OF THE THESIS

The main objective of this investigation was to elucidate the genetic factors underlying ectopic calcification.

Families from Terceira Island, Azores, were investigated. These families seem to have either DISH or Chondrocalcinosis and, sometimes, the association of both. Through linkage study we will try to establish if this disease is a novel form of pyrophosphate arthropathy or a simple association of two different diseases with extremely high prevalence in this island of the Azores archipelago. As it has been previously described, there are many cases of familial CC reported in the literature but there are very few cases of familial DISH and the identification of pyrophosphate dihydrate crystals in this condition is not commonly seen.

This work will also try to clarify the possible role of genes such as *ANKH* and *ENPP-1* in familial Chondrocalcinosis reported in Slovakian pedigrees. Due to the small number of members (affected and unaffected) in each pedigree it was impossible to undertake a linkage analysis approach.

Objectives

- 1) Identify and characterize the clinical and radiological manifestations of DISH and Chondrocalcinosis Families
- 2) Investigate HLA haplotypes present in Azorean families with this disorder
- 3) Perform linkage and recombination mapping in Azorean families
- 4) Screen candidate genes in Slovakian pedigrees with familial CC.

With the completion of this thesis we will hopefully elucidate some genetic factors involved in diseases characterised by ectopic calcification.

3. PATIENTS, MATERIALS AND METHODS

Two sets of patients were used in this thesis: Azorean families, with diagnosis of DISH and CC, in which extensive radiological, biochemical and genetic research was performed; Slovakian pedigrees, with diagnosis of CC, in which candidate genes were selected for sequencing.

3.1 AZOREAN DISH/CC FAMILIES

3.1.1 Selection of families and organising the data

Twelve cases of severe early onset disease were identified. Three individuals with sacroiliac disease and severe enthesopathic spinal disease were identified from the EPOS study radiological cohort [299]. The remaining 9 individuals were identified through a record review conducted at the Rheumatic Diseases Clinic, Hospital of Santo Espírito of Angra do Heroísmo (HSEAH), The Azores.

Probands and all family members were evaluated with a clinical interview and examined for evidence of arthritis by qualified rheumatologists (JBA, MAB).

After obtaining written informed consents, peripheral blood was collected for biochemical studies and DNA extraction. The ethics committee of HSEAH approved all the procedures.

DNA was stored at 4°C for immediate use and at -20°C for backup storage.

All the radiological, biochemical and genetic data were compiled on an Access database.

3.1.2 Radiological study and diagnosis criteria

Radiographs were obtained from axial spine, pelvis, knees, wrists, hands and elbows, in all the study participants (N=92). Other appendicular joints were x-rayed if there were signs or symptoms of disease, and pelvic CT scans performed in cases of radiographic sacroiliac disease. All X-rays were observed by

experienced rheumatologists and radiologists. Affection status was determined radiographically, with the diagnosis of DISH being made according to the Utsinger criteria [172]. The diagnosis of CC was based on the classical radiological evidence of deposition of calcium in the fibrocartilage.

3.1.3 CPPD crystals identification

Synovial Fluid (SF) was collected from the individuals that developed acute arthritis attacks or chronic effusions, from the Azorean families. All samples were collected from knee joints and were examined under compensated polarized light microscopy to check for the presence of CPPD crystals. The presence of other crystals was not investigated, thus, their occurrence cannot be excluded [300].

3.1.4 Biochemical data

All individuals were screened for secondary causes of CC using appropriate standard biochemical tests - serum calcium, iron, magnesium, phosphorus, glucose, uric acid, AST, ALT, alkaline phosphatase, ferritin, transferrin, LDH, cholesterol, triglycerides, urea, creatinine and thyroid hormones (FT3, FT4, TSH), hGH and PTH.

3.1.5 Genetic data

Detection of *HFE*, *TFR2* and *FPN1* gene mutations was performed using the ViennaLab Haemochromatosis Strip Assay [301].

Microsatellite marker studies were carried out in order to exclude already known chromosomal areas linked to familial chondrocalcinosis.

Whole genome scan was performed with highly polymorphic microsatellites from linkage mapping set V.2 (LMSv2, Applied Biosystems).

Selected cases with sacroiliac involvement were genotyped for HLA-B27 [302]. Individuals, randomly chosen, from all families were typed for HLA class I and II alleles to check for associations.

3.2 SLOVAKIAN CC FAMILIES

Twenty five individuals from 8 different pedigrees were used in this study. Interestingly, some of the families herein investigated are descendents of the first familial cases of chondrocalcinosis reported in the literature [303].

We did not have access to any information regarding the radiological and biochemical data of the slovakian pedigrees. Thus, these pedigrees were evaluated based on the assumption that the phenotype showed by the affected individuals was similar to the ones reported on the literature by Zitnan and Sitaj [272, 304].

DNA isolation was performed by salting out from peripheral blood samples that were stored at -20°C at the Wellcome Trust Centre for Human Genetics (Oxford, UK).

3.3 MOLECULAR METHODS

3.3.1 DNA isolation

Genomic DNA was isolated by a standard “salting out” procedure, from peripheral blood lymphocytes. In brief, the protocol used consisted on:

1. Lysis of the erythrocytes with Lysis Buffer;
2. Obtaining a clean (whitish) pellet of mononuclear cells;
3. Lyse mononuclear cells with Tris-EDTA-NaCl, SDS (10%) and Proteinase K;
4. Incubate overnight (approximately 18h) at 42°C with slow agitation;
5. Precipitate the proteins with NaCl (6M);
6. Precipitate the DNA with 90% Ethanol (EtOH);
7. With the help of a pipette tip, tip out the DNA;
8. Wash the DNA with 70% EtOH (temperature of -20°C);
9. Remove the DNA to a 1.5ml eppendorff tube;

10. Allow the EtOH to evaporate completely and resuspend the DNA in 1ml TE buffer.

3.3.2 DNA quantification

DNA quantification was performed on a spectrophotometer GeneQuant II (Pharmacia Biotech). Dilution factor of 50 was used: 2 µl of DNA were resuspended on 98 µl of TE. DNA concentration was estimated using the following formula:

$$\text{DNA Concentration (}\mu\text{g/ml)} = (\text{OD 260nm} \times \text{Absorbance factor of dsDNA (50)} \times \text{Dilution}) / 1000$$

Samples were diluted to 10ng/µl and stored at 4°C for immediate use and at -20°C for longer storage.

3.3.3 HLA typing

HLA loci from class I (A, B, Cw) and class II (DRB1 and DQB) were typed by PCR-SSP (Sequence Specific Primers) using Olerup SSP™ Low Resolution Kits. Number of wells (reactions) is variable for these loci. For each reaction (10 µl) it was used 4.92 µl of deionised water, 3 µl of PCR Solution (containing Buffer and magnesium), 2 µl of DNA (20ng/µl) and 0.08 µl (0.4U) of Taq Polymerase (Roche).

Samples were amplified by PCR using the following thermal cycling parameters: 94°C/2min; 94°C/10sec – 65°C/60sec (10 Cycles); 94°C/10sec – 61°C/50sec – 72°C/30sec (20 cycles); 4°C/∞.

PCR products were then subjected to electrophoreses on a 1.7% agarose gel (Siekem), stained with ethidium bromide (Roche), at 160V for 20 minutes to verify the presence or absence of specific PCR products. Conjugation of positive reactions indicates the allele.

3.3.4 Haemochromatosis mutations typing

The kit used for Hereditary Haemochromatosis (HH) mutations identification in genes HFE, TFR2 and FPN1 is based on PCR-SSO (Sequence Specific Oligonucleotides) and reverse hybridization (ViennaLab). Procedure includes three steps: 1) DNA isolation; 2) PCR amplification using biotinilated primers; 3)

hybridization of amplification products to a test strip containing immobilized allele-specific oligonucleotide probes. Bound biotinylated sequences are detected using streptavidin-alkaline phosphatase and color substrates.

The assay identifies 18 mutations associated with HH: 12 mutations in HFE gene (V53M, V59M, H63D, H63H, S65C, Q127H, P160delC, E168Q, E168X, W169X, C282Y, Q283P), 4 mutations in the TFR2 gene (E60X, M172K, Y250X, AVAQ594-597del) and 2 mutations in the FPN1 gene (N144H and V162del).

Reactions are set up with 15 µl of amplification mix (supplied by the manufacturer), 5 µl of DNA (diluted to 15ng/µl) and 5 µl of Taq Polymerase (diluted to 0.2U/µl). Samples are amplified using the following PCR thermal cycling program: 94°C/2min; 94°C/15sec - 58°C/30sec - 72°C/30sec (35 cycles); 72°C/3min; 4°C/∞.

After amplification PCR products are analyzed on a 1.7% agarose gel stained with ethidium bromide. Ten µl of amplified products are then mixed with 10 µl of a denaturing solution (DNAT) on the typing tray. Hybridization to strips was performed on an AutoLipa apparatus, according to the manufacturer's instructions, with solutions supplied with the kit.

Interpretation of the results is made with a schematic drawing supplied with the kit. Positive reactions indicate a normal, heterozygous or homozygous individual.

3.3.5 Microsatellite amplification

All microsatellite amplification was performed using the Applied Biosystems Prism Linkage Mapping Set Version 2 (LMSv2) marker Set.

Microsatellites were amplified in 20 µl reactions with 2 µl of (10 x) Gold Buffer, 0.4 µl of MgCl₂ (0.5mM), 0.5 µl of dNTPs (0.2mM each nucleotide), 0.7 µl of primer Mix (0.35pmol), 0.1 µl of TaqGold (0.5U) and 10 µl of DNA at a concentration of 10ng/µl (100ng). Volume was complete with deionized water.

PCR's were performed with the following thermal cycling: 94°C-5min; 94°C-1min - 50°C/55°C/60°C-1min - 72°C-1min (30 Cycles); 72°C - 10 min; 4°C - ∞.

3.3.6 Microsatellite pooling

To simplify the genotyping process PCR amplified products were pooled, according to the fluorochromes and allele size range expected, avoiding overlapping alleles.

96-well plates, were prepared by adding a mixture of 10 µl of Hi-Di formamide (Applied Biosystems) and 0.3 µl of internal line standard GeneScan 400HD-ROX (Applied Biosystems) to each well. After adding the pooled PCR products, plates were denatured, at 94°C for 2 minutes, and then immediately run on AB Automated DNA analysers - 3700 and 3130XL.

3.3.7 Genotyping

Products were sized using the program GENESCAN v.1.1 (Applied Biosystems) and genotypes semiautomatically assigned using GENOTYPER V1.1 (Applied Biosystems). GENEMAPPER software V4.0 was also used for product sizing and genotypes assignment.

Genotypes were analysed using GeneMapper Software Version 4.0 from Applied Biosystems.

3.3.8 Statistical methods

3.3.8.1 Mendelian errors and inconsistencies

To minimize errors extensive checking procedures were undertaken. Mendelian errors and inconsistencies were manually detected using GENOTYPER and GeneMapper (Applied Biosystems).

GAS v2.0 was used to convert size data into discrete allele numbers.

PEDCHECK [305] was used to detect marker typing incompatibilities in pedigree data.

3.3.8.2 Parametric and non-parametric analysis

Parametric single point analysis using the program Vitesse was performed (assuming autosomal dominant transmission, a penetrance of 95% and an allele frequency of 0.1%).

Regions with indication of linkage were analysed with parametric multipoint and haplotype analysis. MERLIN and GENEHUNTER Plus were used for parametric and non-parametric linkage analysis.

Family based association tests were performed using FBAT software [306].

3.3.9 Sequencing

Sequencing reactions of genes *ANKH* and *ENPP1* were performed only on Slovakian pedigrees.

LEMD3 was sequenced in one Azorean Pedigree diagnosed with Osteopoikilosis.

All sequencing reactions were performed with BigDye Terminators (Applied Biosystems) V.3.1 and V1.1. Sequencing reactions were resuspended in milliQ water or HiDi formamide and run on either ABI 3700 or 3130XL Automated DNA Auto Analysers.

3.3.9.1 *ANKH*

ANKH mutations were assessed by direct sequencing of promoter and coding regions using primers located in flanking intron sequences (table 9).

Table 9. Primers used in PCR reactions and sequencing (primers kindly provided by Dr Yun Zhang, Wellcome Trust Centre for Human Genetics)

ANKH Region	Forward Primer	Reverse Primer
Promoter 1	CAAGCCCCCAGACCGTCCCCG	TTCTGCTGACAGCGGCTCCAT
Promoter 2	CGCCCGCCCTGATTTCCTC	TCTGCTGCCGCGAGGGGACT
Promoter 3	GAGCAGCCGCGCTCGGAGAA	GAAGTCGATGGCTATGTTGG
Exon 1	AGATGTGTGTGGGGTCAGC	GCTGGAAAAGTCACCCCTTGA
Exon 2	ACCCTATAAGCTACTTAGTG	AGAAACATTTGATAATTAGTG
Exon 3	TGACCCACTCTGGGTAGAGG	CCTGCCATTAAGCTGTACACAC
Exon 4	TGCAGTCCTTCACTCCACTG	CAGTTACACACGCCAGAAGG
Exon 5	CCCCTGTGCTTCTGTCACTG	CTGTCTTTCCCTGCAGACATC
Exon 6	GGCTTGTGATTAAACATACGAAGG	CACCGAACGAGATCTTTATGG
Exon 7	CGTGCTTCGTACCTACTGT	CCCCAACGTCACATTAACT
Exon 8	GCCCGAGAGACACTCAACAT	TGCCCTTTACAAAAACCAA
Exon 9	AGCAAGCAGGTGGCAGATCTG	AGTGAAATTTTACATTTGTG
Exon 10	ACCCTGGCAGGAAGATGAG	CCCAAACCTCCCTGACAACAT
Exon 11	GAAGCCAGCAGATGGAGAAC	ACCATCCAACCTGGTCACTG
Exon 12	AGAATGAATAAGGCACGGGACG	ACAACGTCAACCGTGAGGCAG

PCR reactions were optimized for all primer and set up to 50 µl with: (10x) PCR Gold Buffer; 200 µM of each dNTP; 1, 2 or 3 mM of MgCl; 0.4mM of Primer Mix and 1U of Ampli TaqGold (Applied Biosystems). Deionized distilled water was used to attain the final volume. Annealing temperature and magnesium concentration were adjusted until maximum specificity was reached for each set of primers.

PCR amplification was performed using the following thermal cycling conditions: 94°C-14min; 94°C-1min, 50-60°C-1min, 72°C-1min (35 cycles); 72°C - 10 min; 4°C - hold.

PCR products were purified in Millipore MANU3050 filter plates on a vacuum manifold. Purified products were resuspended on water.

Sequencing reactions of 20µl were set up using: 2 µl of Ready Reaction Premix (2.5x) with BigDye Terminators V.3.1; 2µl of BigDye Seq Buffer (5x); 1µl of Primer and 5µl of Purified PCR Template. Deionized distilled water was used to attain the final volume.

Sequencing reactions were subjected to the following thermal cycling programme: 96°C - 5sec; 96°C - 10sec, 50°C - 5sec, 60°C - 4min (25 cycles).

Sequencing reactions were purified using Corning Glass Fiber Filter Plates following the Protocol: one volume of sequencing reaction was mixed with 2 volumes of 6M potassium iodide and this mixture was then transferred to

Corning 0.25mm Glass fiber plate. The wells were filled with 200 µl of 80% ethanol (Sigma, Molecular Biology Grade) and centrifuged at 2000xg for 1 minute. Flow through was discarded. Ethanol wash was repeated. The plate was allowed to dry for 1 min. 40 µl of MilliQ sterile water was added to each well and incubated for 1 minute. Elution of sequencing reactions into a new collection plate was performed by centrifuging (2000xg) for 2 minutes.

Sequences were compared using FACTURA and SeqScape (Applied Biosystems).

3.3.9.2 *ENPP1*

ENPP1 mutations were assessed by direct sequencing of promoter and coding regions using primers located in flanking intron sequences (see table 10).

Table 10. Primers used in PCR reactions and sequencing (primers designed and kindly provided by Dr Yun Zhang, Wellcome Trust Centre for Human Genetics)

<i>ENPP1</i> Region	Forward Primer 5' - 3'	Reverse Primer 5' - 3'
Exon 1	GCCAAGGATCTGACCGCGAG	TAGGGCACTTGCAAAGGCTG
Exon 2	ACATTTGGAGAAATAAAAGG	TCACATTCTTAATAAGGCTG
Exon 3	CCCATCTGTATTTTCTAAAG	AACGTGTATGTATGCTAAAC
Exon 4	CATGGTAGTGGCAGATTCTG	TTCAGCTAATATAGTTGGCC
Exon 5	TATTGATTATACACCTCCAG	AAAAAATGATGCTGTACTTC
Exon 6	AGATCACACAGACCTTAGTG	GAGTAATCACACTATTAAAG
Exon 7	GATTTGTATCCACAGTGTTG	ACTACCTATTGGAAAATAAG
Exon 8	TTGCGTTTTGCCATTACCTG	TTACAAAAGAATGGCCACAG
Exon 9	TTGTCATCTAAGTGCTGAAG	TCAGTTCTCATTTTTTCAGCC
Exon 10/11	GCATTTAGTAGCTCTTAATG	TAAAATGGTCTAGCAGCCTC
Exon 12	ATAGCCATTAGTGTGGAAGG	TCAGAAAAAATTGGGAAACC
Exon 13	TTACCTATCAGATCTTCAAC	AGGAAGGTATTCTTAATATG
Exon 14	CTCTTTAAAACCTGCCTTGG	CCCAGATTACTAAAATATTC
Exon 15	AGCAATTTCAAGAAAAGTTG	AGAGAATCATACTTATACAG
Exon 16	TGGTTTTATTACCGTTGTG	CATTCAAGACATATAATCAC
Exon 17	TCTTATCTTATCATAACCAG	TCAGGAAGAAGGTAAAAATC
Exon 18	TAAATGACTCATGTATTTGG	TCACAAAAGATCTACATGCG
Exon 19	TGATCTTTGTTCTATGTTGG	AACTGTCAAGCAAAGAAACC
Exon 20	GTTTATGCTTCTACCCCTTG	AACTGAGTATAAAGCCAAGG
Exon 21	AAGATGAATATACTAGTAGG	ACCCTATAATGTATGGTAGG
Exon 22	ACTTGGGAAAGTTTACAGG	GTGTACTAGTATTCTCAAAG
Exon 23	ATGCTAATTTTGAATCATGG	GTCATACACATAAACATACG
Exon 24	ATTAAAAGCATGCTCTACTG	AGTCCATGTGTACTCAGTTG
Exon 25-1	ATGATGCCTAACAAATCATG	CAATTTTAACCGTCTATAGG
Exon 25-2	AAACTGTGCGACCAGAGTTAG	TTATGAGAATTTTAAAGTGG

PCR reactions were optimized for all primer sets and 20 µl were setup with: (10x) PCR Gold Buffer; 200 µM of each dNTP; 1, 2 or 3 mM of MgCl₂; 0.4mM of Primer

Mix and 1U of Ampli TaqGold (Applied Biosystems). 100 ng of DNA was added to each reaction. Deionized distilled water was used to attain the final volume. Annealing temperature and magnesium concentration were adjusted until the maximum specificity was reached for each set of primers. Primer set for Exon 1 required the utilization of BSA (3%) and DMSO (10%).

PCR amplification was performed using the following thermal cycling conditions: 94°C-14min; 94°C-30sec, 50-60°C-30sec, 72°C-1min (35 cycles); 72°C - 15 min; 4°C - hold.

PCR products were purified either in Millipore MANU3050 filter plates, by centrifugation (PCR products resuspended in MilliQ water), or by ExoSAP-IT (GE Healthcare) according to the manufacturer's instructions.

Sequencing reactions of 20µl were set up using: 2 µl of Ready Reaction Premix (2.5x) with BigDye Terminators V.3.1; 2µl of BigDye Seq Buffer (5x); 1µl of Primer and 5µl of Purified PCR Template. Deionized distilled water was used to attain the final volume.

Sequencing reactions were then subjected to following thermal programme: 96°C - 5sec; 96°C - 10sec, 50°C - 5sec, 60°C - 4min (25 cycle).

Sequencing reactions were purified using Sephadex G50 Superfine Gel (Amersham) on Millipore Multiscreen Plates (MAHVN4550) following the Protocol: load 400 µl of Sephadex G50, dissolved in MilliQ water, in each well of the filter plate and centrifuge at 4000rpm for 5 min. Discard the water and load total sample; spin at 4000rpm for 5 minutes and collect the sample on another plate.

Sequences were compared using SeqScape (Applied Biosystems) and BLAST.

3.3.9.3 *LEMD3*

LEMD3 mutations were assessed by direct sequencing of promoter and coding regions using primers located in flanking intron sequences (see table 11).

Table 11. Primers used in PCR reactions and sequencing (primer sequence kindly provided by Dr Jan Hellemans, University of Ghent)

LEMD3 Region	Forward Primer 5' - 3'	Reverse Primer 5' - 3'
Exon 1.1	CAGCGCGGTCTAGTTCAGG	GCTGTTAGTCACCTCGCTGG
Exon 1.2	GAGGAAAGACCGGGCTTCG	AGGAACGGGGCTGGAGTCC
Exon 1.3	GAGCCGAAACCTCGAAGAGG	AGCGCGGCTAAAGCGGACC
Exon 1.4	CCGCTGACTGCTAATCGGC	GCCTGTTGACGAATCCAAGG
Exon 1.5	TGTGATCAAGTGGACTCCAGC	TTCGGGTTCCGAAAGCTTCGG
Exon 2	GTTTTAGCAAAGTACATGCTGG	TTAGTTGATAGTCATTCTCATGC
Exon 3	ATTTGTCCTTCAGATTATGTGGC	CACAAATATAACACTGGACTTGG
Exon 4.1	TTCTTCTGTTTAAGAGAGTACTG	AAACATATGATTCTTACAAGTAGC
Exon 4.2	ATTTCTTTGTTTATATTCTTACTGG	CTGCTCATATTATACCAAACAGC
Exon 5/6	AAGGATACTTTACAGAGAGTCG	AATACACACAAAAATTCAGGCTGC
Exon 7/8	GAAGGTTCAATCCGTTGTGGC	CTCTCAATATAATTTTCAATACTGC
Exon 9	AACAGCTAGAGTTAACTATTACC	GAATTGTACTGTTTCACTTAACAC
Exon 10	GAATGAACTGAATGATTGAATACC	GAATATCATGCATTTTCCAGCC
Exon 11/12	GAGACAGTGAAGAATTTTACCG	CTAAATTCTTTTATTTGAAATCTGG
Exon 13.1	CTTTTACCACAGTTAATTTTCTGC	GAACCTTAAGACTTCTGGAACG
Exon 13.2	CTTTTACCACAGTTAATTTTCTGC	TCGCTTATAAGCAACAGAAACC

PCR reactions were optimized for all primer sets and 25 µl were setup with: (10x) PCR Buffer; 200 µM of each dNTP; 1, 2 or 3 mM of MgCl; 0.4mM of Primer Mix and 2U of GoTaq (Promega). 100 ng of DNA was added to each reaction. Deionized distilled water was used to attain the final volume. Magnesium concentration was adjusted until the maximum specificity was reached for each set of primers.

PCR amplification (GeneAmp PCR System ®9700) was performed using a Touchdown Programme to maximize the yield of specific products. Method used had the following thermal cycling conditions: 94°C-15sec, 65°C-30sec (20 cycles); 94°C-15sec, 55°C-30sec (10 cycles). The initial annealing temperature (65°C) incrementally decreases by an additional 0.5°C in each of the first 20 cycles, followed by 10 cycles at 55°C.

PCR products were purified using Microcon Spin columns (Millipore) according to the manufacturer's instructions.

Sequencing reactions of 10µl were set up using: 2 µl of Ready Reaction Premix (2.5x) with BigDye Terminators V.1.1; 1µl of BigDye Seq Buffer (5x); 0.16µl of Primer and 2µl of Purified PCR Template. Deionized distilled water was used to attain the final volume.

Sequencing reactions were then subjected to following thermal programme: 96°C – 5sec; 96°C – 10sec, 50°C – 5sec, 60°C – 4min (25 cycle).

LEMD3 Sequencing reactions were purified using an Ethanol/EDTA/Sodium acetate precipitation according to the following protocol: 1 μ l of 125mM of EDTA and 1 μ l of 3M sodium acetate were added to each well after which 25 μ l of 100% ethanol were also added. Plate was sealed, inverted and incubated at room temperature for 15 minutes. After centrifuging at 4000rpm for 30 minutes, with a temperature of 4°C, it was inverted and briefly centrifuged at 1000rpm. 35 μ l of ethanol 75% were then added. The plate was centrifuged at 3000rpm for 15 minutes, inverted and briefly centrifuged to remove excess ethanol. After complete ethanol evaporation samples were either stored at -20°C or resuspended on Hi-Di formamide for immediate capillary electrophoresis. Sequences were compared using SeqScape (Applied Biosystems).

4. RESULTS

4.1 ECTOPIC CALCIFICATION IN THE AZORES – CLINICAL AND RADIOLOGICAL MANIFESTATIONS OF DISH AND CHONDROCALCINOSIS FAMILIES

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The results herein presented are part of the article presented as annex 1 [173].

Keywords: DISH, chondrocalcinosis, ectopic calcification

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ABSTRACT

Introduction: 12 families, multiply affected with DISH and/or CC, were identified on the island of Terceira, the Azores, supporting the hypothesis that both disorders share common aetiopathogenic factors.

Patients and Methods: 103 individuals from 12 unrelated families were assessed. Probands were identified from patients attending the Rheumatic Diseases Clinic - Hospital de Santo Espírito, Angra do Heroísmo. Family members were assessed by rheumatologists (JBA, MAB) and radiologists (KC, EGM). 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.

Results: Ectopic calcifications were identified in 70 patients. Axial pain, elbow, knee and MCPs pain and/or swelling, deformity and radiographic enthesopathic changes were the most frequent findings/symptoms. Elbow and MCPs periarticular calcifications were observed in 35 and 5 patients respectively, and CC was identified in 12 patients. Fifteen patients had sacroiliac disease on CT scans – 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. The mean age for symptoms to develop was 38 years.

Conclusions: 1) These families may represent a familial type of pyrophosphate arthropathy with a phenotype that includes peripheral and axial enthesopathic calcifications; 2) The concurrence of DISH and CC suggests a shared pathogenic mechanism in the two conditions.

INTRODUCTION

Diffuse idiopathic skeletal hyperostosis (DISH, Ankylosing Hyperostosis, Forestier-Rotes Querol's disease, MIM 106400) is a common condition amongst the elderly characterized by ossification of the anterior longitudinal ligament (ALL) affecting, in particular, the right side of the spine with preservation of the intervertebral disc space. Whilst spinal involvement in DISH is nearly universal, in some cases peripheral entheses may also be involved [191]. Studies have reported that oochronosis, hypophosphatasia, acromegaly, haemochromatosis, hypomagnesaemia, fluorosis, and hypervitaminosis A, may also cause similar enthesopathy [227].

DISH is more frequent in males and its prevalence increases with age, affecting mainly subjects over the age of 40 [172]. It may be as prevalent as 25% in males and 15% in females over 50 years of age [230]. However, accurate measures of the disease prevalence are not well established, as large radiographic studies would be required because of the high prevalence of asymptomatic disease.

There have been few systematic studies of the clinical consequences of DISH [307]. One of the first reports on this condition found that stiffness and mild middle to low back pain were found in 80% of patients, dysphagia was present in 28%, elbow pain in 13% and heel problems in 23% [217]. Peripheral nerve entrapment has also been described [308].

It has been reported that 50% of DISH cases are complicated by a similar condition, ossification of the posterior lateral ligament (OPLL, MIM 602475) [206] suggesting that they share common aetiopathogenic factors. OPLL is mostly found in Asian populations, in particular amongst Japanese, where it has been reported to have a prevalence of 1.9 to 4.3% [212].

The aetiology of DISH is unknown, with pathogenic roles of degenerative, inflammatory and metabolic factors (in particular diabetes mellitus) having been suggested [172, 175].

Chondrocalcinosis (CC), a common cause of joint pain and arthritis particularly in the elderly, is characterized by the deposition of calcium containing crystals in articular cartilage, synovial membranes and, less often, in periarticular soft tissues [309, 310]. The physicochemical mechanisms that cause this deposition

are still unknown, but a key role of disordered inorganic pyrophosphate (PPi) metabolism is suspected [311]. The disease is most commonly seen as a sporadic form in elderly people with a prevalence of 25% in the population aged 85 years and 3% in people aged between 65 and 69 years [312]. CC is often associated with endocrine and metabolic diseases such as haemochromatosis and hypophosphatasia among others [313]. A hereditary form of “chondrocalcinosis articularis” was first reported by Zitnan and Sitaj [272] in seven Czechoslovakian kindreds in 1963. Since then, many reports of familial aggregation have been reported in different ethnic groups most of them showing an autosomal dominant inheritance [253, 273, 314-318]. In contrast, very few cases of familial DISH have been reported [170, 319-321] and, to our knowledge, there is only one description of DISH in association with calcium pyrophosphate dihydrate (CPPD) crystals [206].

Reports of familial CC and shoulder arthropathy suggests that disordered PPi metabolism may play a role in calcific rotator cuff diseases [322]. It has also been suggested that diffuse spinal ligamentous calcification could be mediated by an increased PPi generation by ligament fibroblasts encasing the spine [323]. Although many factors are likely to be involved in the regulation of calcification and ossification processes, studies of CC and DISH have implicated control over extracellular PPi levels as one of the most important factors in their aetiopathogenesis [311].

In this study we report the clinical, radiological and some genetic aspects of families with ectopic calcifications from Terceira Island, characterised by enthesopathic musculoskeletal calcifications, fulfilling frequently the radiological criteria for DISH, and in some cases associated with CPPD chondrocalcinosis. The Azores islands are located in the middle of the Atlantic Ocean, and were very important in the 15th and 16th century sea traffic between Europe and America. The islands were officially populated in 1439. European colonisation of the archipelago was surprisingly swift since all the main current towns already existed by 1550. Portuguese documents show that the islands were populated after 1432 by Portuguese, Dutch (Flemish), Spaniards, Berbers, Jewish and Italians. In addition black African slaves were brought to the islands. Presently, the Azores archipelago is an autonomous Portuguese region with approximately 250,000 inhabitants [324].

PATIENTS AND METHODS

Twelve cases of severe early onset disease were identified. Three individuals with sacroiliac disease and severe enthesopathic spinal disease were identified from the EPOS study radiological cohort [299]. The remaining 9 individuals were identified through a record review conducted at the Rheumatic Diseases Clinic of Hospital of Santo Espírito, Angra do Heroísmo, The Azores. Probands and family members were interviewed and examined for evidence of arthritis by qualified rheumatologists (JBA, MAB).

Radiographs were obtained from axial spine, pelvis, knees, wrists, hands and elbows, in all the study participants (92). Other appendicular joints were x-rayed if there were signs or symptoms of disease, and pelvic CT scans performed in cases of radiographic sacroiliac disease. All X-rays were observed by rheumatologists (JBA, MAB) and radiologists (KC, EGM). Affection status was determined radiographically, with the diagnosis of DISH being made according to the Utsinger criteria [172]. The diagnosis of Chondrocalcinosis was based on the classical radiological evidence of deposition of calcium in the fibrocartilage.

All individuals were screened for secondary causes of CC using appropriate biochemical tests - serum calcium, iron, magnesium, phosphorus, glucose, uric acid, AST, ALT, alkaline phosphatase, ferritin, transferrin, LDH, cholesterol, triglycerides, urea, creatinine and thyroid hormones (FT3, FT4, TSH), hGH and PTH.

Detection of *HFE*, *TFR2* and *FPN1* gene mutations was performed using the ViennaLab Haemochromatosis Strip Assay [301]. Microsatellite marker studies were carried out in order to exclude already known chromosomal areas linked to familial chondrocalcinosis.

Synovial Fluid (SF) was collected from the majority (n=12) of the individuals that developed acute arthritis attacks or chronic effusions. All samples were collected from knee joints and were examined under compensated polarized light microscopy to check for the presence of CPPD crystals (GHB,VC). The presence of other crystals was not investigated, thus, their occurrence cannot be excluded [300]. Selected cases with sacroiliac involvement were genotyped for HLA-B27 [302].

RESULTS

Pedigrees

The extended families of all 12 probands were identified, and those living in The Azores invited to participate (n=115 individuals). Of these, 103 individuals (70M: 33F) were studied in entirety. Ten young asymptomatic individuals were not x-rayed, and family members without complete clinical, biochemical or radiological assessment were also excluded (2 individuals).

The mean pedigree size was 8.6 ± 3.3 (Min=5; Max=17). Five pedigrees consisted of 2 generations, and the remaining 7 pedigrees had 3 generations; three to eleven affected individuals were identified in each family. Observation of pedigrees showed a pattern of transmission compatible with an autosomal dominant monogenic disease (table X). No generation was skipped and about 50% of descendents of an affected parent were similarly affected irrespective of parent or offspring gender. Eight probands were born in the same village.

Clinical data

The mean age at which symptoms developed was 37.8 ± 12.9 years (Min = 15: Max = 60); only 7 patients (10%) developed symptoms after 50 years of age, and 15 patients (21%) were symptomatic before 30 years of age.

Lumbar, dorsal and/or cervical pain was the most frequent axial symptom (Table 2). Back pain was “inflammatory-like” in several patients - pain and stiffness were worse during the night or in the morning, improving with movement, and worsening again at the end of the day. The meaning of this symptom was not clear, and we do not know if there is a relationship with the sacroiliac disease identified in 15 patients (21%). Only some of these patients had inflammatory-like lumbar pain, and the radiological investigation of younger patients with inflammatory-like axial pain could not identify sacroiliac disease on CT scans.

Elbows, knees, and MCPs were the most frequently affected peripheral joints (Table 2), and symptoms usually started in the same decade as back pain. Strikingly, elbow pain (59%) or deformity (49%) was identified in all families, and elbow pain was often the initial manifestation of disease.

Co-occurrence of other disorders with the ones investigated in this study were reported. Two patients, one female and one male, had a previous clinical, serological and radiological diagnosis of Rheumatoid Arthritis, but enthesopathic calcifications were also identified in the peripheral or axial skeleton. Three members of family 1, two males and one female, had the radiological diagnosis of Osteopoikilosis; two members of family 2 had the diagnosis of bilateral Osgood-Schlatter disease, and 13 members of six families reported previous nephrolithiasis, including one family with five affected members. Pseudogout occurred in 4 patients.

Radiological data

Ectopic calcifications were identified by x-rays in 70 patients (67%), Males = 40: Females = 30 (Table 1); mean age 59.3 ± 15.1 years (Min = 31: Max = 87). Of these, 12 patients were asymptomatic (Mean age 47.6 ± 7.8 years; Min = 36: Max = 63).

According to the Utsinger criteria [172], 52 patients (M=29: F=23; 74%) could be classified as definite (12; M=7: F=5; 17%), probable (18; M=9: F=9; 26%), and possible (22; M=13: F=9; 31%) DISH. Twelve patients (17%; M=8: F=4), had calcification of knee fibrocartilage and/or calcification of the triangular ligament of the wrist, and 14 (20%) had radiology findings insufficient for a definite diagnosis. A diagnosis of concomitant DISH and CC was made in 12 patients.

1. Axial skeleton

Spinal x-ray abnormalities were identified in 57 patients (83%) and ranged from exuberant osteophytes and syndesmophyte like spurs to, enthesopathic calcifications that were identified as continuous or discontinuous smooth or massive calcifications along the anterior and/or lateral aspect of vertebral bodies.

Bilateral or unilateral sacroiliac ankylosis was identified on x-rays and confirmed by CT scan in 11 patients, and bilateral sclerosis (ilium and/or sacrum) in another 4 patients. In two cases with early fusion, bridging syndesmophytes could be seen anterior to the sacroiliac joint, with no evidence on CT scan of joint erosion. In established ankylosis, cortical remnants of the sacroiliac joints were visible in some cases, having not been eroded as part of the disease process.

2. Appendicular skeleton

Periarticular, capsular or synovial calcification (50%), and ulnar insertion calcification of the triceps tendon (47%) were the most frequent findings, and frequently bilateral. These calcifications were usually more exuberant with increasing age of patients, and in some cases, calcification and bone fragmentation were striking, making impossible to define the limits of elbow structures.

Superior or inferior patella ligament calcification (29%) and cartilage calcification (16%) were the most frequent radiological findings. The current mean age of individuals with knee calcification was 74 years (74.4 ± 8.1). CPPD crystals were identified from knee effusions in 10 patients from 8 different families. MCP joints, mainly the 2nd and 3rd, were frequently painful (40%) or deformed (24%), and x-rays showed subluxation of metacarpal heads, sclerosis, narrowing of joint space, periarticular calcifications, and metacarpal head osteophytosis (30%), in various proportions. Radiocarpal compartment disease was identified in 9 patients (13%). Eight patients had triangular ligament calcification, and another patient had bilateral exuberant hyperostosis of the radial extremity. Hip disease was identified in 7 male patients and one female. Only one patient (definitive DISH) had been submitted to surgery, twenty years before, and x-rays now show extensive heterotopic ossification with restrictive hip motion.

Thirty-three individuals from eleven families (32%; M=19: F=14) had no ectopic calcifications. Three had pseudogout. One of them, a 36 years old female, had bilateral knee effusion and CPPD crystals were identified in the synovial fluid.

Biochemical data

Biochemical tests revealed normal values of serum calcium, iron, phosphorus, magnesium, potassium, uric acid, AST, ALT and alkaline phosphatase.

Ten male individuals (11%) had ferritin values higher than 300 $\mu\text{g/l}$ with an average value of 596 $\mu\text{g/l}$ and ranging from 333 to 1200 $\mu\text{g/l}$. Five had transferrin saturation (TS) higher than 44% and only 3 had *HFE* mutations.

One individual was a compound heterozygote (C282Y/H63D), another was H63D homozygote and the other was H63D heterozygote. Only one woman had a

ferritin value higher than 200 µg/l. No mutation was found in this case and the TS value was normal. All patients had repeated measurements of serum ferritin which confirmed the high values first identified, and were not associated with the erythrocyte sedimentation rate or with the disease activity.

DISCUSSION

It is not yet known if this disorder occurs with the same prevalence in the other islands of the archipelago. However, in Terceira Island, this condition may possibly represent an endemic and common condition. Since 8 probands were born in the same village there is a possibility of a genetic founder effect.

Periarticular calcifications were observed on elbows and MCPs x-rays, in a large group of patients. Furthermore, calcification of knee fibrocartilage and/or triangular wrist ligament was observed in 17% of patients, and pseudogout episodes and CPPD crystals were identified in the majority of the families investigated. Although we have not investigated for the presence of other crystals, the identification of CPPD crystals in all synovial fluids examined suggests that these families have a form of autosomal dominant CPPD deposition disease (OMIM 118600); however unlike previous reports of this condition, in these families the CC was in all cases complicated by peripheral and axial enthesopathic calcifications.

It is also possible that the phenotypes observed in these families may correspond to the association of two common diseases in this population, DISH and pyrophosphate arthropathy. Familial DISH has previously been reported [319, 320], and it is known that if this condition coexists with rheumatoid arthritis or gout, more exuberant new bone formation is observed [325].

In some cases we cannot exclude that the CC may have developed as a complication of haemochromatosis, as 10 out of 92 cases had high ferritin and TS levels. However, it is unlikely that this could be responsible for more than a small proportion of cases, as other individuals had normal iron stores. Previous studies have demonstrated the rarity of haemochromatosis in this island [326]. Furthermore, the clinical and radiological features seen in this disorder are, in many aspects, different from the arthropathy of haemochromatosis [313].

Despite the numerous reports of familial CC since its first description by Zitnan and Sitaj [272], the prevalence of the familial form of CC is still unknown. Studies of late onset CC suggest that the condition has low familiarity [255]. Throughout two years of investigation in this island we came across several other cases with similar phenotypes (data not shown) in which familial aggregation could not be investigated because their family members had already died or emigrated.

Previous family studies on CC describe mainly two different clinical phenotypes; early onset with polyarticular involvement with variable severity of the arthropathy (OMIM 600668), and another phenotype characterized by late-onset oligoarticular CC with arthritis resembling sporadic disease [251, 327]. The early onset phenotype was observed in all the Azorean families described in this study, with initial symptoms starting in the 3rd - 4th decades in the majority of the family members.

Classical descriptions of ankylosing hyperostosis of the spine and CC [217] draw attention to distinctive radiographic abnormalities. Axial skeleton calcification, mainly of the ALL, has been usually ascribed to DISH, and is more frequent in the anterolateral dorsal spine. However calcification of spinal ligaments can also occur as a complication of CPPD deposition disease. In spinal CC (CPPD) the initial site of crystal deposition appears to be the outer fibres of the annulus fibrosus which may produce opaque linear or tumoral densities that resemble the syndesmophytes of ankylosing spondylitis (AS) [328]. Furthermore, widespread involvement of the annulus and the nucleus pulposus may occur, especially in patients with familial disease, producing extensive disk calcification. Whilst most of our cases had no radiographic disc abnormalities, in some cases disc calcification was observed. Whether our cases have disseminated CPPD deposition causing their spinal disease is the subject of current post-mortem studies.

Previous reports have highlighted the resemblance of spinal CPPD, DISH and AS [171, 314, 328] and it can be difficult in some cases to differentiate these conditions. This may be one of the explanations for the higher percentage of HLA-B27 negative AS that has been reported in some populations [329]. Some of the patients reported here have previously been diagnosed as seronegative AS, and even retrospectively we could not be sure of the underlying diagnosis in one case. These findings suggest that a proportion of B27-negative cases of AS may represent an ossifying enthesopathy distinct from the immune-mediated process involved in AS.

Sacroiliac bilateral or unilateral complete ankylosis was identified in 11 patients, and bilateral sclerosis (ilium and/or sacrum) in another four. The first descriptions on sacroiliac disease in DISH concludes that capsular ossification is frequent even early in the disease, but complete bridging of the joint is not reached before the sixth decade [330]. Durbach et al [331] described asymmetric

superior intraarticular partial fusion, osteophytes with or without bridging, and vacuum phenomenon, while Weisz and Green [332] reported progressive obliteration of the SI joints in 4 of 11 patients with longstanding Forestier`s disease. DISH primarily affects the superior and ligamentous part, leaving unaffected the lower 2/3 part of the sacroiliac joint, which is a synovial component in the normal SI joint. Sclerosis or erosions of the SIJ have been described in CC [333], and calcification in the articular cartilage of both the ilium and sacrum was noted in a postmortem examination study [252]. Thus, both DISH and CC can cause sacroiliac disease, and both may be relevant in the sacroiliac ankylosis or sclerosis identified in our patients.

CPPD deposition is thought to be a consequence primarily of raised extracellular PPI levels, which are known to reduce the formation of hydroxyapatite crystals. The frequent coexistence of both crystal types in synovial fluid samples from pseudogout cases has not yet been properly explained. Identification of the genetic mechanism operating in these families may provide insights into this apparent conundrum.

Osteopoikilosis (MIM 166700) is an autosomal dominant skeletal dysplasia that has been described in association with several conditions; it is characterised by the presence of multiple hyperostotic areas in the metaphyses and epiphyses of the long bones, carpus, tarsus and membranous bone [334]. The association of Osteopoikilosis with DISH or CC is, to our knowledge, first reported in one of our families; the relevance of this finding is not known but deserves further investigation. A loss-of-function mutation in *LEMD3* (MIM 607844), a gene that is involved in BMP and TGF- β signalling, coding for an integral protein of the inner nuclear membrane, has recently been identified [335].

These Azorean families with their multiple clinical and radiological manifestations support the presence of a common aetiopathogenic pathway involved in pyrophosphate arthropathy and DISH. Identifying the genetic causation of this disorder is likely to add to our currently limited understanding of the aetiopathogenesis of these common disorders of ectopic calcification.

4.2 ECTOPIC CALCIFICATION IN THE AZORES – A PALEOPATHOLOGICAL STUDY

The coexistence of Ankylosing Spondylitis and Diffuse Idiopathic Skeletal Hyperostosis – a post-mortem diagnosis

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The results herein presented are part of the article presented as annex 2 [205].

Keywords: Spondyloarthropathy; Syndesmophytes; Joint fusion; Sacroiliitis; Bone pathology.

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ABSTRACT

A 72 years old male was diagnosed as having Ankylosing Spondylitis, mainly according to radiological findings, and confirmed as HLA-B27 negative. Post-mortem examination of the skeleton raised doubts on the initial diagnosis, since spinal findings pointed out also to Diffuse Idiopathic Skeletal Hyperostosis. The authors discuss the differential diagnosis and enhance the post-mortem findings which allowed the diagnosis of the two clinical entities in the same patient.

INTRODUCTION

Ankylosing Spondylitis (AS) and Diffuse Idiopathic Skeletal Hyperostosis (DISH) are both complex disorders of unknown etiology, involving mainly the axial skeleton with bony proliferation and ankylosis, although both can also display enthesopathic manifestations in the peripheral skeleton. Despite these similarities, AS is a rare chronic inflammatory disorder (0.23-1.8% in Europe) of earlier age onset and with inflammatory manifestations, whereas DISH is usually described as a more common skeletal condition (6%–12% of routine autopsies) of elderly patients, with minor clinical manifestations [336-341]. Calcium Pyrophosphate Dehydrate Crystal deposition disease (CPPD CD) is another condition which involves the axial spine, being frequently undiagnosed. The most known finding is disc calcification, but syndesmophytes and apophyseal joint space narrowing, sclerosis and ankylosis are also frequent findings [342, 343].

The aim of this paper is to report a patient with sacroiliac and spinal disease, diagnosed as AS late in life, and confirmed as HLA-B27 negative. X-rays findings in the axial skeleton could also allow a DISH diagnosis. The authors had the rare opportunity to examine the skeletal remains years after the patient's death. The differential diagnosis is discussed, taking into account the new information obtained.

CASE REPORT

Sacroiliac and spinal disease was identified in a 72 years old male from Terceira Island (Azores, Portugal) during the European Vertebral Osteoporosis Study radiologic cohort [344]. He and his family members were further interviewed and examined for evidence of arthritis at the Rheumatic Diseases Clinic of the Hospital de Santo Espírito (Angra do Heroísmo, Terceira Island). This patient had been a farmer since a young age, and his main complaints were related with ischaemic heart disease, which was the cause of death when he was 78 years old. He had dorsal and lumbar back pain, at least since he was 40 years old, but he was unable to properly characterize his back pain. He had no peripheral joint disease, uveitis, psoriasis or inflammatory bowel disease (IBD). Anteroposterior spine and pelvis X-rays suggested a diagnosis of Ankylosing Spondylitis, mainly because bilateral fusion of the anterior and posterior components of the sacroiliac joint. His brother, an 80 years old male, had dorsal spine DISH on X-rays, but no sacroiliac disease. The patient had four offspring, three males without symptoms and a 46 years old daughter which had bilateral sacroiliac sclerosis and bilateral enthesopathic tricipital calcification. She had episodes of dorsal inflammatory-like pain, although she also complained of dorsal non-inflammatory pain. Routine blood tests of the patient and family members were normal, and HLA-B27 typing resulted negative.

Several years after the patient's death, the skeletal remains were morphologically examined by paleopathology experts, and post-mortem X-rays and CT scans were taken. Post-mortem examination was made using the complete skeleton. No particular findings were observed in the appendicular skeleton, namely periostitis, erosions, or enthesopathic phenomena, but several findings were observed in the axial skeleton.

On pelvis examination, bilateral sacroiliac joint fusion and para-articular bony bridging were confirmed. Furthermore, X-rays showed a severe bilateral and symmetric sacroiliitis, and the CT study showed intra-articular ankylosis and erosion of the whole sacroiliac joint.

The anterior aspect of the spine displayed a flowing and undulating calcification along the anterior and right lateral aspect of T6-L5 vertebral segments. Furthermore, bony bridging between C4-C5 was observed. The intervertebral disc

height was normal in all segments and there was no involvement of the discovertebral junctions.

Degenerative disease and ankylosis of some apophyseal and costovertebral joints were observed in the posterior aspect of the spine. Specifically, CT images showed intra-articular apophyseal joint fusion between C4-C5, T7-T8 and T9-T11. Intra-articular costovertebral joint fusion in T10 and T11 was identified on the right side. Additionally, there was calcification of interspinous and supraspinous ligaments between T6-T11.

In addition to the morphological examination, several bone samples including the calcified anterior longitudinal ligament were examined in a JEOL JSM-6301F Scanning Electron Microscopy (SEM) that was coupled to a Noran Voyager X-ray elemental microanalyser (XRM) with EDS (Energy Dispersive Spectrometry) detection system, thus allowing *in situ* elemental analysis.

The SEM imaging was used to detect crystal structures in the bone samples. Several calcium crystal shapes are reported in the literature [342], including pin-like, rod-like, granular, or rectangular, but none of these were observed in the bone samples. An elemental analysis of different regions of the ligament was also performed. Figure 2 shows one of these analyses, for which the relationship calcium/phosphorus was 1.73 ± 0.04 , confirming the absence of calcium pyrophosphate crystals (expected value for calcium pyrophosphate – 1.0). All the other spectra confirm a ratio of 1.70 ± 0.08 .

DISCUSSION

Although bilateral sacroiliitis is a strong radiological evidence of AS, sacroiliac joint fusion may occur in both AS and DISH, even if ankylosis of the synovial compartment of the sacroiliac joints is rarely observed in DISH [336, 337, 345-347]. DISH para-articular osteophytes may simulate intra-articular bone fusion on X-rays of DISH patients, and they are easily identified with sacroiliac CT scan [348].

The calcification or ossification encompassing the annulus, and the anterior longitudinal ligament, which involves mainly the right side of the dorsal spine, are the classic features of DISH. The preservation of inter-vertebral disc space height, which is another classic finding in DISH, is sometimes unhelpful in the differential diagnosis mainly in old patients with concomitant osteoarthritis. In AS, calcifications or ossifications within the peripheral portion of the annulus fibrosus and erosive vertebral abnormalities are the main findings, although in longstanding cases the anterior longitudinal ligament may become involved. Furthermore, the involvement of the apophyseal and costovertebral joints and the interspinous and supraspinous ligaments, are frequent findings in AS [336, 337, 340, 341, 347, 348].

The ante-mortem data available in this case was not enough to help us to unambiguously establish the diagnosis. The patient was observed for the first time when he was 72 years old, and was not able to remember his past symptoms. X-rays and CT scans were observed by several experts and a diagnosis of idiopathic AS was made, although he was not HLA-B27 positive. The association of this allele with AS is at least 95% [338, 349]; although this association is not as strong as with other spondyloarthritis, the patient had neither Psoriasis nor Inflammatory Bowel Disease.

According to ante and post-mortem data, a diagnosis of coexistence of AS and DISH was proposed in our case. The absence of peripheral arthritis and of CPPD crystals did not support the diagnosis of CPPD CDD disease. Sacroiliac, apophyseal and costovertebral ankylosis, and calcification of the interspinous and supraspinous ligaments were favorable to the diagnosis of AS. The absence of involvement of the discovertebral junctions and the flowing calcification of the anterior longitudinal ligament in the dorsal and lumbar spine are very frequent in

DISH. This association is quite rare mainly because AS is not a common disease, and very few cases have been reported in the literature until now [341, 350]. Other family members had a DISH diagnosis, and although there are few reports of familial DISH, a recent report [340] shows that in Terceira Island families with multiple members affected with DISH and/or chondrocalcinosis are common.

Sacroiliac CT scan allowed the confirmation of intra-articular bony fusion, and the findings observed in the post-mortem examination (confirmed by CT scans) were crucial to diagnose this association. The coexistence of DISH and AS may be possible mainly if the patient is diagnosed in a geographic area where DISH has a high prevalence.

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4.3 ECTOPIC CALCIFICATIONS IN THE AZORES – GENETIC ASPECTS OF DISH AND CHONDROCALCINOSIS FAMILIES

Manuscript in preparation

GENETIC ASPECTS OF DISH AND CHONDROCALCINOSIS FAMILIES

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Keywords: Linkage analysis, ectopic calcification, genetics, Azores

ABSTRACT

Twelve families with early onset of calcium pyrophosphate dehydrate (CPPD) chondrocalcinosis (CC) and diffuse idiopathic skeletal hypersostosis (DISH) were identified in Terceira Island, the Azores, Portugal. The interest in this disorder results from its unusual and exuberant radiological characteristics, its disabling potential at a young age and the fact that it appears to be monogenic.

After clinical and radiological characterization, 92 individuals, from 12 unrelated families, were selected for a whole genome linkage study. DNA was extracted and HLA typing was carried out in probands and several family members; hereditary haemochromatosis mutations were screened and microsatellite amplification was performed using LMS V.2. Mendelian inheritance errors were checked using PEDCHECK. Parametric and non-parametric linkage analysis was performed using GENEHUNTER and MERLIN.

No association with HLA alleles was seen; haemochromatosis mutations were identified in 31 individuals from 9 families. Suggestive linkage to an area of chromosome 16 (16q12.1-16q22.1) was obtained in the whole genome analysis performed.

Although the molecular basis of this disorder has not been totally clarified, this study showed that these families were not associated to chromosomal areas previously linked to either DISH or CC. Through the analysis of pedigrees this disorder seems to be monogenic. Thus, the results of this study suggest the involvement of a possible new major gene in the aetiopathogenesis of this disorder.

INTRODUCTION

Chondrocalcinosis (CC) is an important and frequent cause of joint pain and arthritis, characterized by the deposition of calcium crystals in the synovial fluid, cartilage and, less often, the periarticular soft tissue [309] [351]. The characteristic radiological appearance of CC is associated with the deposition of calcium pyrophosphate dihydrate (CPPD) crystals in the joint cartilage. CPPD-CC can be idiopathic (sporadic) [352], familial [353] or secondary to numerous metabolic disorders [275]. Prevalence and incidence of each form of CPPD-CC are difficult to ascertain, as a result of its high clinical heterogeneity technical difficulties with crystal identification [354]. Idiopathic CPPD-CC is the most common, affecting 3-7% of the population 65 to 70 years old, increasing drastically to 25-60% by the age of 85 years [250, 352]. Most cases of CPPD-CC are isolated; but, since the first report of a familiar form of CPPD-CC [353], designated as “chondrocalcinosis articularis”, many familial reports followed [[355],[356],[317],[357],[358],[359],[360],[361],[362],[363],[364],[274]]. The majority suggested an autosomal dominant pattern of transmission with variable penetrance.

Two chromosomal regions have been linked to CPPD-CC: CCAL1[MIM 600668] on human chromosome 8q and CCAL2, on chromosome 5p [MIM 118600] [277]. The disease-causing gene in CCAL1 was never mapped but there is a strong possibility that this gene is primarily related to osteoarthritis (OA) and that the CPPD deposition is secondary, enhanced by the degenerative changes in cartilage. The identification of the disease causing-gene on the CCAL2 locus on chromosome 5p was achieved after the cloning of the gene for progressive ankylosis (*ank*) in a mouse model. Mutations at this locus cause a generalized progressive form of arthritis accompanied by mineral deposition, formation of bony outgrowths and joint destruction [84]. *ANKH* (CCAL2) is, for the moment, the only monogenic cause identified for CC [278].

Diffuse idiopathic skeletal hyperostosis (DISH) is a common condition amongst the elderly, characterized by ossification of the anterior longitudinal ligament and peripheral entheses affecting, in particular, the right side of the spine with preservation of the intervertebral disc spaces. DISH is the main non-inflammatory ossifying enthesopathy involving not only the spine but also peripheral joints [365]. The aetiology of DISH is still unknown. The possible pathogenic role of

degenerative, inflammatory and metabolic factors has been suggested [172]. Oochronosis, hypophosphatasia, acromegaly, haemochromatosis, hypomagnesaemia, fluorosis, and hypervitaminosis A, may all cause similar enthesopathic changes [227].

Data suggests that DISH is more frequent in males and that prevalence increases with age, affecting mainly subjects above age 40 years [172]. It may be as prevalent as 25% in males and 15% in females over 50 years of age [230]. Half of DISH cases are associated with the ossification of the posterior lateral ligament (OPLL) [206], suggesting these share common aetiopathogenic factors. OPLL is mostly found in Asia, particularly amongst Japanese, where it has been reported to have a prevalence of 1.9 to 4.3% [366]. A large-scale study of OPLL was performed to identify new susceptibility genes for OPLL and re-examine previously reported associations; 35 candidate genes were genotyped in sporadic OPLL patients (n=711) and controls (n=896). Significant associations were found in three genes, the most significant being with *TGFB3* ($P=0,0004$). Association with *COL11A2*, *ENPP1* and *TGFB1* was not reproduced in this study [248].

There are genetic and clinical links between CC and DISH. There is some evidence that disordered pyrophosphate metabolism may play an important role; in general, conditions that favour the increase of inorganic pyrophosphate (PPi) promote CPPD crystal formation [268]. There is evidence of genetic involvement in CC and forms of spinal ossification in both humans [367] and animal models; the *ank* mouse develops severe hydroxyapatite chondrocalcinosis and spinal ossification [31], and the tip-toe walking (*ttw*) mouse is of human OPLL [165].

Our main objective was to elucidate the genetic factors underlying a recently reported rheumatic disorder characterised by DISH and the co-occurrence of CPPD-CC [368]. Previous studies in one of these pedigrees suggested a genetic region not linked to previously known chromosomal areas associated with either CC or DISH. We enlarged the number of individuals investigated trying to determine if this disease is a novel form of pyrophosphate arthropathy or a simple association of two different diseases with extremely high prevalence in this island of the Azores.

PATIENTS, MATERIAL AND METHODS

Patients and families

Twelve pedigrees, with 92 individuals examined (affected, unaffected) were included for the linkage analysis; another 51 individuals, were ascertained by family history. Most of these individuals were deceased or inaccessible and to all was ascribed an unknown status. The structure of the pedigrees analysed can be seen in Figure 1.

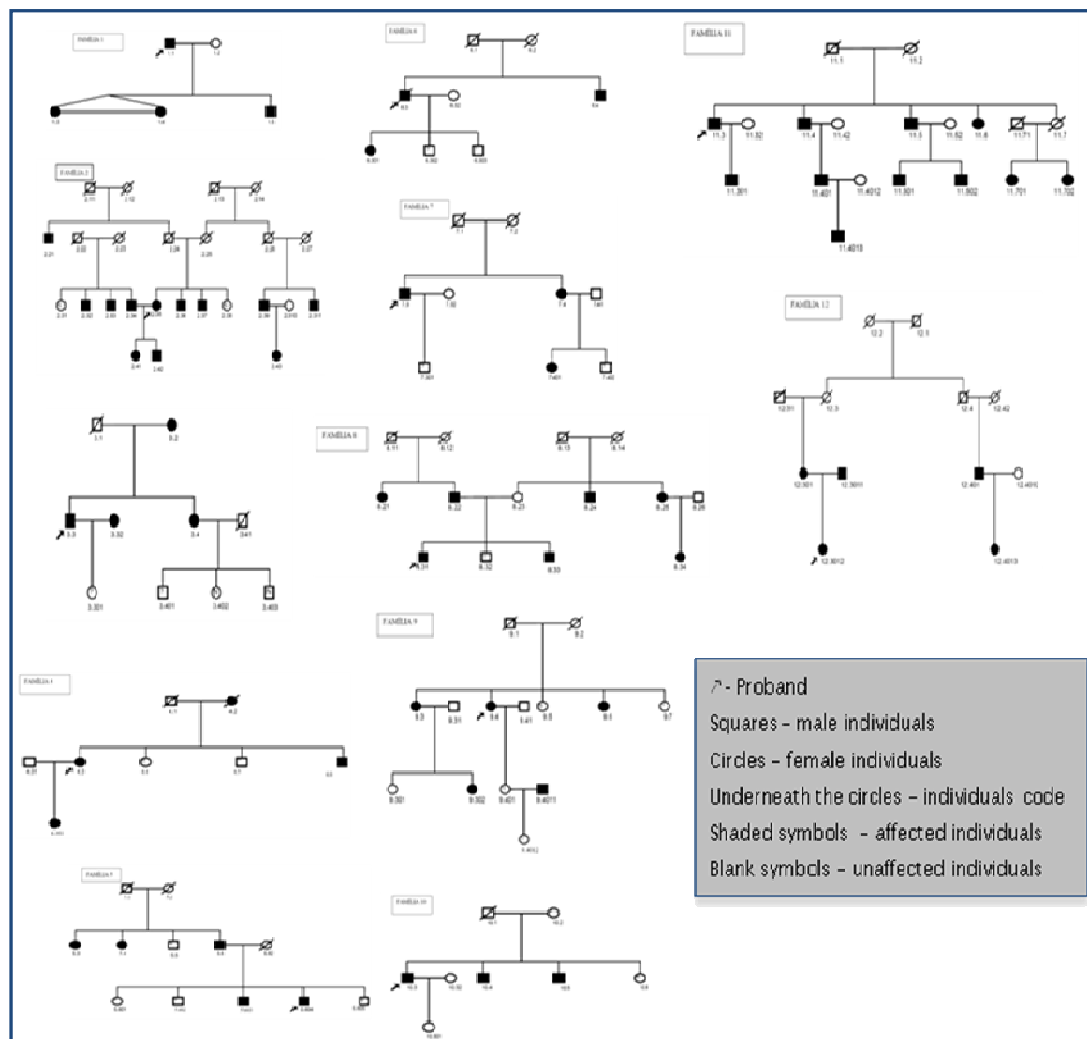


Figure 1. Azorean kindreds included in the study

Methods

After informed consent, peripheral blood was collected for biochemical analysis and DNA isolation. DNA was extracted by salting-out, and quantified using a spectrophotometer. HLA typing (class I and II alleles) from probands, some affected individuals and other family members was performed by PCR using sequence-specific primers (Olerup SSP) [369].

To exclude secondary CC associated with haemochromatosis a full assessment of iron metabolism was performed. Levels of serum iron, ferritin, transferrin, total iron binding capacity (TIBC) and transferrin saturation (TS%) were investigated in all 92 individuals under study. Due to high transferrin saturation in a few individuals, hereditary haemochromatosis mutations were screened in genes *HFE*, *TFR2* and *FPN1* were screened using the Haemochromatosis Strip A^{ssay} (ViennaLab), following the manufacturers instructions [370].

Highly informative microsatellite markers from Linkage Mapping Set V2 (Applied Biosystems) were used for linkage analysis. Microsatellites were amplified in 20 µl reactions with 2 µl of (10 x) Gold Buffer, 0.4 µl of MgCl₂ (0.5mM), 0.5 µl of dNTPs (0.2mM each nucleotide), 0.7 µl of primer Mix (0.35pmol), 0.1 µl of TaqGold (0.5U) and 10 µl of DNA at a concentration of 10ng/µl (100ng). Volume was completed with deionized water. Amplification was performed in the following conditions: 94°C-5min; 94°C-1min - 50°C/55°C/60°C-1min - 72°C-1min (30 Cycles); 72°C - 10 min; 4°C - ∞. To simplify the genotyping process PCR products were pooled, according to the fluorochromes and allele-size range expected, avoiding overlapping alleles. 96-well plates, were prepared by adding a mixture of 10 µl of Hi-Di formamide (Applied Biosystems) and 0.3 µl of internal line standard GeneScan 400HD-ROX (Applied Biosystems) to each well. After adding the pooled PCR products, samples were denatured, at 94°C for 2 minutes, and immediately run on ABI automated DNA genetic analysers - 3700 and 3130XL. Fragment analysis was semiautomatically performed using GENOTYPER software (Applied Biosystems) and GeneMapper Software Version 4.0 (Applied Biosystems).

To minimise data errors, extensive checking procedures were undertaken. GAS (version 2.0) was used to assign discrete allele numbers to alleles. Mendelian inheritance was checked manually with GENOTYPER and GeneMapper (Applied Biosystems). PEDCHECK [305] was used to screen all data for previously

undetected Mendelian inconsistencies. Statistical analysis, parametric and non parametric analyses were performed using VITESSE [371], GENEHUNTER and MERLIN [372]. Marker positions were obtained from public databases. Parametric linkage analysis was performed assuming autosomal dominant transmission, penetrances of 90 to 95%, phenocopy rates of 0.05 to 0.1% and minor allele frequencies of 0.1% to 1%.

RESULTS

Autosomal dominant inheritance

The phenotype under study showed strong familial aggregation and suggested an autosomal dominant mode of transmission. This is corroborated by the following observations: (i) all affected individuals examined have at least one affected parent with complete penetrance in recent generations; parents not evaluated (deceased) were considered to have unknown affection status; (ii) males and females were similarly affected (66 affected: 29 females, 37 males); (iii) males and females both transmit the disease to their offspring and there are several cases of male-to-male transmission; (iv) the disease does not appear in the descendants of unaffected individuals.

HLA Typing

A total of 58 individuals (44 affected and 14 unaffected or possibly affected), randomly selected, were typed to investigate possible associations with HLA alleles. The allelic frequencies of these individuals were similar to the ones found in general population. Chi-square analysis did not show any significant differences.

Haemochromatosis mutations screening

Nine pedigrees had individuals with either the H63D or C282Y *HFE* mutations. No mutations were identified in genes *TFR2* and *FPN1*.

Although all the 92 individuals were screened, only the ones with TS levels higher than 50% will be presented (table 2). From the 92 individuals in which iron metabolism was evaluated 15 (16%) had TS higher than 50%; 13 of these individuals (87%) were affected; 7 of the 15 (47%) did not carry any of the mutations tested; 3 (20%) were H63D homozygotes; 4 (27%) were H63D heterozygotes and one was a compound heterozygote (C282Y/H63D).

Pedigree	Serum iron	Ferritin	Transferrin	TS%	HH genotype	Affection status
1	99	333	222	54%	WT / WT	Affected (Proband)
2	82	693	65	117%	WT / WT	Affected
2	124	259	244	62%	WT / WT	Affected
4	153	318	268	92%	WT / WT	Affected
4	115	71	282	51%	H63D / H63D	Unaffected
8	131	128	258	66%	WT / WT	Affected
9	113	321	230	61%	WT / WT	Unaffected
10	179	257	288	77%	WT / WT	Affected
10	129	702	256	64%	C282Y / H63D	Affected (Proband)
11	114	546	202	87%	H63D / WT	Affected (Proband)
11	113	144	206	73%	H63D / WT	Affected
11	185	639	270	141%	H63D / H63D	Affected
12	110	99	213	65%	H63D / WT	Affected
12	119	121	273	52%	H63D / WT	Affected
12	114	55	221	63%	H63D / H63D	Affected

Table 2. Serum iron, ferritin, transferrin, TS% and HFE genotypes in individuals with TS% above 50.

Linkage Analysis

A whole genome scan, including the 92 individuals from the 12 pedigrees (Fig.1) was performed with 189 microsatellite markers with an average distance between markers of 17cM, on all autosomes. Parametric and non parametric analyses were performed. Parametric LOD scores (Table 3) were generated by Genehunter Plus using a minor allele frequency (MAF) of 1%, a phenocopy rate of 0.05%, and a penetrance of 90%.

Chromosome	PARAMETRIC MLS		NONPARAMETRIC MLS	
	Single	Multi	Single (Analyze)	Multi
1	0,00	-0,32	0,21	0,29
2	0,00	-1,76	0,10	0,05
3	0,00	-0,04	0,07	0,19
4	0,00	0,31	1,13	0,93
5	0,00	0,35	0,57	1,79
6	-2,60	-3,70	0,33	0,45
7	0,28	0,16	0,21	1,27
8	-2,64	-1,38	0,06	0,20
9	-1,73	-1,34	1,14	1,17
10	-0,10	-0,46	0,34	0,50
11	-1,75	-1,72	0,12	0,20
12	-0,30	-2,46	0,23	0,57
13	-2,74	-2,24	0,09	-0,14
14	-2,07	-3,37	0,00	-0,13
15	-4,21	-	0,00	-
16	0,73	1,04	2,38	1,16
17	-0,75	-1,69	0,26	0,18
18	-1,12	-2,99	0,12	0,14
19	-1,24	0,08	0,01	0,89
20	-2,78	-2,71	0,00	-0,69

21	-0,20	-0,07	1,24	1,57
22	-0,96	-2,75	0,56	0,22

Table 3. Results from the analysis with 189 markers in all autosomal chromosomes

Because the average distance between markers was higher than 10cM, one extra set of 137 markers, distributed on all autosomes, was further amplified and reanalyzed to enlarge the power of the study. Parametric and non-parametric analyses were performed with MERLIN [372]. A total of 327 microsatellites were included in the analysis. Owing to its complexity, pedigree 2 was excluded from the analysis due to computation time and memory constraints. Parametric analysis assuming an autosomal dominant mode of inheritance with a MAF of 0.1%, phenocopy rate of 0.1% and penetrance of 90% did not provide any positive result. The most important single-point non-parametric results, using MERLIN, can be seen in table 4.

Chromosome	Position (cM)	Zmean	LOD*	p-value
8	154.00	1.86	1.18	0.01
16	49.50	1.42	0.68	0.04
16	67.62	1.35	0.85	0.02
19	76.15	1.33	0.85	0.02

Table 4. Single point non-parametric results (MERLIN) using 327 microsatellite markers on 11 pedigrees. *LOD score corresponds to Kong and Cox LOD score.

Non-parametric multipoint analysis, using GENEHUNTER, was then performed on chromosomes 8, 16 and 19. When analyzed all together there was no sign of linkage. Some of the pedigrees, however, showed very weak signs of linkage when analyzed independently, but none of them was significant.

Chr.	Pedigree	Marker	LOD	NPL	p-value	Information
8	5	D8S1771	1.42	2.03	0.13	0.933
16	1	D16S3136	0.60	2.32	0.06	1.000
16	1	D16S415	0.60	2.32	0.06	1.000
16	5	D16S404	1.31	1.77	0.13	0.420
16	5	D16S3068	1.42	2.17	0.08	0.703
19	1	D19S414	0.60	1.55	0.06	0.500
19	10	D19S571	0.61	1.45	0.06	0.522

Table 5. Results of non-parametric multipoint analysis (GENEHUNTER) on chr. 8, 16 and 19.

Because there was again, although weak, suggestive linkage to an area of chromosome 16, a new set of 13 microsatellite markers, covering this entire

chromosome with an average spacing of 8.99 cM, were further amplified. Through haplotype analysis it was possible to see that not all the pedigrees were linked to this area. With the objective of making the group of pedigrees more homogeneous, the ones that were not linked to this area were withdrawn from the analysis with the following results:

Chromosome 16	Location	Z-mean	p-value	delta	LOD	pvalue
Markers	cM	Max=-3.39	Max=0.000	Max=0.696	Max=5.62	Max=0.000
D16S521	1.08	0.33	0.4	0.117	0.03	0.4
D16S3027	8.71	0.43	0.3	0.150	0.05	0.3
D16S423	14.05	0.42	0.3	0.158	0.05	0.3
D16S418	14.77	0.49	0.3	0.184	0.06	0.3
D16S3075	23.28	0.48	0.3	0.286	0.10	0.2
D16S3046	40.65	1.09	0.14	0.539	0.45	0.08
D16S3100	52.26	1.83	0.03	0.696	1.32	0.007
D16S415	67.62	1.38	0.08	0.696	0.86	0.02
D16S3057	77.13	0.33	0.4	0.271	0.06	0.3
D16S514	81.15	0.10	0.5	0.072	0.00	0.4
D16S515	92.06	0.80	0.2	0.471	0.26	0.14
D16S516	95.45	0.36	0.4	0.229	0.06	0.3
D16S505	108.96	0.52	0.3	0.557	0.20	0.2

Table 6. Results from singlepoint analysis (MERLIN) on chr. 16

DISCUSSION

The first clinical description of CPPD-CC was reported by McCarty *et al.* in 1962 [352]. This is a common pathological finding, characterised by the accumulation of CPPD crystals in articular and periarticular tissues, with a prevalence that increases drastically with age [373].

CPPD-CC is clinically heterogeneous, including: 1) an asymptomatic presentation, common in elderly people, affecting mainly the knee by the deposition of CPPD crystals in articular hyaline and fibrocartilage (CC);[374],[375]; 2) an acute form of CPPD arthropathy, known as pseudogout, typically monoarticular but sometimes subacute and/or polyarticular 3) a chronic progressive CPPD arthropathy known as pseudo-osteoarthritis, affecting multiple joints with or without acute inflammatory reaction, usually bilateral and symmetrical [376]; 4) pseudo-rheumatoid arthritis, which is less common and characterised by bilateral and symmetrical polyarthritis, with low-grade inflammation lasting weeks or months; 5) a miscellaneous pattern mimicking ankylosing spondylitis, among other disorders; 6) a tophaceous pseudogout, resulting from the deposition of calcium pyrophosphate crystals as large soft tissue masses [377].

The variable clinical presentation of CC, which was observed in this study tends to concentrate in the families, thus suggesting genetic heterogeneity. No chromosomal region was found in clear linkage in the affected families. Several reasons, can be hypothesized to explain these results: 1) the phenotypic heterogeneity observed within and among pedigrees can result from genetic heterogeneity; 2) the disorder may also have other modes of inheritance, including a recessive one, because most pedigrees come from a particular village of the Island, where the disease may be very frequent; 4) unknown environmental factors can also play a role on phenotypes and are somewhat causing difficulties in the analysis.

HLA association in DISH and CC has been reported since 1976 [378, 379]. Shapiro and colleagues [379] reported association between HLA-B27 and Forestier's disease. After analysing 47 patients the authors found that 16 were HLA-B27 positive (34%), hypothesizing this gene could be involved in new bone formation, due to the association with ankylosing spondylitis (AS) and DISH, where bone proliferation is an essential feature [380]. This was followed by many other that

rejected this association of HLA alleles and DISH [381]. There were conflicting results [382-387] originating reports of other associations such as: HLA-B8 [388] and HLA-B5 [389].

Nyulassi *et al.* reported an association of HLA alleles A2 and Bw35 in Czechoslovakians of Hungarian ancestry with CC [390]. Reginato studied the HLA alleles in Chiloe islanders with ankylosing chondrocalcinosis, in 1979. None of the alleles segregated with the disease and no allelic association was found [316].

Rodrigues-Valverde *et al.* did not find any significant differences in the distribution of HLA-A and HLA-B antigens while investigating 13 spanish pedigrees with familial chondrocalcinosis. None showed segregation of HLA haplotypes with the disease. Similarly to Nyulassi *et al.*, HLA-A*02 and HLA-B*35 allele frequencies were quite high. These are, however, similar to those found in the general population from Terceira island. In addition, none of the other alleles have shown significant differences when compared with the general population of Terceira.

Several probands show similar haplotypes. Although interesting, this can be explained by common founders, since several are originating from the same small village. High resolution typing would help to assess the level of shared HLA alleles.

As can be seen in table 3, there was only one individual with the mutation C282Y. Most individuals with high TS% were not homozygotes for C282Y which is the main HFE haplotype related to haemochromatosis. Ferritin levels were also quite below 1000 ng/ml, the level associated with haemochromatosis. We do not think that haemochromatosis is causing the phenotype although it is possible that it may modify it, in a few specific cases.

The region on chromosome 16 that shows low linkage, in all the analyses performed, is a relatively large area of approximately 15cM. The duplication of this exact same region, creating a gene dosage imbalance, has recently been associated with spina bifida [391].

Chromosome 16 has a total number of 1392 genes; the chromosomal area with suggestive linkage, from 16q12.1 to 16q22.1, comprehends 252 genes. There

are several genes of interest, in this region, among which, the most attention-grabbing are:

- i. *HAS3*: (16q22.1): encodes the hyaluronan synthase 3, a membrane-bound enzyme which is involved in the synthesis of the unbranched glycosaminoglycan hyaluronan, or hyaluronic acid, a major constituent of the extracellular matrix. Interestingly, there are numerous reports associating the use of intra-articular hyaluronan with the development of CPPD arthritis [392-394];
- ii. A cluster of cadherin genes which encode calcium-dependent adhesive proteins that mediate cell-to-cell interaction and are involved in the structural and functional organization of cells in various tissues [395].
- iii. *SLC12A3*, which is quite important in ion homeostasis, and mutations of which can cause Gitelman Syndrome (GS) [OMIM 263800]. GS is an autosomal recessive form of familial hypokalaemia and hypomagnesaemia [396]. There are many studies reporting the familiar association of GS with CC due to CPPD deposition [397, 398]; this is thought to be resultant from chronic hypomagnesaemia [396].

4.4 OSTEOPOIKILOSIS STUDY IN AZOREAN FAMILIES

A NOVEL LEMD3 MUTATION COMMON TO PATIENTS WITH OSTEOPOIKILOSIS WITH AND WITHOUT MELORHEOSTOSIS.

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The results herein presented are part of the article presented as annex 3 [399].

Keywords: osteopoikilosis, LEMD3,

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ABSTRACT

Objective: to identify putative mutations in the gene *LEMD3* in affected individuals from a multiple affected Azorean family with autosomal dominant osteopoikilosis and patients with osteopoikilosis and/or melorheostosis identified at Oxford.

Methods: all the 13 *LEMD3* exons and flanking splice sites were sequenced among a three-generation family of Azorean origin with three family members affected with osteopoikilosis, and patients from Oxford including a proband with melorheostosis and her father who had osteopoikilosis plus two individual patients with melorheostosis.

Results: we identified a novel heterozygous mutation in exon 8 of gene *LEMD3*. This mutation, a C to T substitute at position 2032bp (cDNA), changes amino acid at 678 from Arginine to a Stop codon, and segregates with the phenotype of all the affected individuals in the Azorean family. In the nuclear family, collected at Oxford, both father and daughter have the exact same mutation. None of the unaffected individuals in family AZ and the healthy controls has this mutation. No *LEMD3* mutations were detected in the other two sporadic melorheostosis patients.

Conclusions: our findings suggest that the *LEMD3* mutation reported here is the cause of osteopoikilosis in the Azorean family and co-occurrence of osteopoikilosis and melorheostosis in the family originary from Northern Ireland. However, since we did not find any mutation in the *LEMD3* in two other individuals with melorheostosis it is probable that other genes may be involved.

INTRODUCTION

Osteopoikilosis (MIM #166700) is an uncommon bone dysplasia, which is most often an incidental finding with no clinical consequence [400]. Albers-Schönberg first described it in 1915 describing this disorder as a rare structural anomaly of the skeleton [401]. In 1916, Ledoux-Lebard et al. introduced the term “*osteópoecilie*”, originating from Greek and meaning “spotted bones” [402]. Other denominations include “disseminated osteopathy” [403] and osteosclerosis disseminata familiaris [404].

This disorder is seen in both men and women and may become evident at any age although its appearance below the age of 3 is uncommon. Familial studies indicate an autosomal dominant pattern of transmission that may become more prominent in each succeeding generation. It is characterized by numerous small, well defined, round areas of increased density, of 2-10 mm in diameter, containing sclerotic bone tissue [405]. The most frequently involved sites are the epiphyses and metaphyses of long tubular bones and the carpi, tarsi pelvis and scapulae [406].

Osteopoikilosis may occur as an isolated anomaly or coincide with cutaneous lesions. A skin manifestation of Osteopoikilosis - *dermatofibrosis lenticularis disseminate* - was, for the first time, described in 1928 by Buschke and Ollendorff. The skin lesions consist of yellow, lenticular infiltrations, which may be grouped, mainly located on the buttocks and thighs, but sometimes also affecting the upper extremities, back and abdominal skin [407].

The association of Melorheostosis in families with autosomal dominant Osteopoikilosis has been reported numerous times [408]. Melorheostosis (MIM 155950) is a sporadic condition characterized by bony sclerosis and soft tissue involvement. The bony sclerosis typically affects the diaphyses of the long bones the carpal and tarsal bones, and the pelvis [408]. It usually affects one side of the body and the lower limb is more frequently involved than the upper limb. Radiologically irregular assymetrical longitudinal cortical hyperostosis is observed in the long bones and this typical radiological appearance is often described as molten wax flowing down the side of a candle [408].

These disorders may coexist in the same individual and such sporadic familial co-occurrence has been often reported in the literature [409]; [410]; [408]. Buschke-Ollendorff syndrome (BOS), another skeletal dysplasia disorder, is an autosomal dominant disorder characterized by disseminated connective tissue nevi of elastic type and osteopoikilosis [411]; [412]. Recently, loss of function mutations in the *LEMD3* (also named as *MAN1*) gene, encoding an inner nuclear membrane protein [413], have been suggested as the cause for the above two disorders and BOS [335].

The objective of our study was to identify putative mutations in the gene *LEMD3* on a multiple affected Azorean family with autosomal dominant osteopoikilosis and in patients with osteopoikilosis and/or melorheostosis identified at Oxford.

PATIENTS AND METHODS

Human subjects. A multiple affected Azorean family (Family AZ) is a three-generation family of Azorean origin. The proband, a 23 year old female, was identified from patients attending the Rheumatic Diseases Clinic - Hospital de Santo Espírito, Angra do Heroísmo. Two other affected individuals were further identified in this family. We have also investigated four patients attending the Oxford Nuffield Orthopaedic Centre. These included a proband with melorheostosis and her father who had osteopoikilosis (Family H, from Northern Ireland origin) - the mother is healthy in this nuclear family, and two individual patients with melorheostosis. All the individuals had a full skeletal radiographic survey. Healthy control subjects, 135 portuguese Caucasians from the Azores and 207 British Caucasians from Oxford, were recruited. Genomic DNA from the above patients and healthy controls was isolated from peripheral blood. Informed consent was obtained for all patients included in this study.

Mutation screening and Genotyping. We conducted mutation screening across all 13 *LEMD3* exons and flanking splice sites via polymerase chain reaction (PCR) amplification, followed by direct sequencing of PCR fragments (ABI Big Dye chemistry), using an ABI 3700 automatic sequencer (Applied Biosystems, UK). We have first sequenced all the members of AZ family, Family H and two individual patients with melorheostosis. After that we have sequenced 24 healthy controls from Oxford, 135 healthy controls from the Azores and another 183 Oxford healthy controls.

RESULTS

We identified a novel heterozygous mutation in exon 8 of gene *LEMD3*. This mutation, a C to T substitute at position 2032bp (cDNA), changes amino acid at position 678 from Arginine to a Stop codon, and segregates with the phenotype of all the affected individuals in Family AZ. In Family H, identified at Oxford, both the daughter and her father have the exact same mutation (but not the healthy mother). None of the unaffected individuals in Family AZ has this mutation. No *LEMD3* mutations, in the coding region or exon-intron boundaries, were detected in the other two sporadic melorheostosis patients. None of the 135 Azorean controls or 207 British Caucasians from Oxford had this mutation.

DISCUSSION

In this study, we report a novel heterozygous mutation in exon 8 of gene *LEMD3* that segregates with disease in two families (AZ and H families). This mutation causes an arginine to a Stop codon substitution at amino acid position 678. None of the controls and healthy members of the families have this mutation suggesting that this variant is not a common polymorphism. Further more, the absence of any other mutations in the *LEMD3* in the affected members in these two families confirms that this mutation is the cause of the disease in the families. Given the fact that, in our study, the individuals with melorheostosis do not have this mutation and that, in Family H, we can see a co-occurrence of osteopoikilosis and melorheostosis leads us to the conclusion that this gene harbours the disease-causing mutation for osteopoikilosis but not for melorheostosis.

Interestingly, we have found the exact same unreported mutation in two, geographically, very distant families. In our perspective this fact can be explained by the Azorean settlement. The Azores Islands (Portugal) are located in the middle on the North Atlantic Ocean and were officially populated in 1439. During the 15th and 16th centuries these islands played an important role in commerce and slave trade between Africa, America and Europe. This strategic position between the three continents brought to Azores people from many different origins, mainly Portuguese but also Jews, Moorish prisoners, African black slaves, Dutch (Flemish), French, Italians, English and Spaniards [414]. It is probable that the individuals from AZ and H Families have related genetic background.

A recent study identified loss-of-function mutations in *LEMD3* in all affected individuals of three osteopoikilosis families and in three unrelated individuals with the disease [335]. All the patients in this report had either osteopoikilosis, with or without BOS, or osteopoikilosis with melorheostosis and BOS. None of the patients was diagnosed as melorheostosis only.

A latest report from the same group identified several other cases with *LEMD3* mutations [415]. 19 new patients with osteopoikilosis, BOS and/or melorheostosis were included in this study. The authors reported that they have further identified 6 mutations in *LEMD3* in 6 patients with osteopoikilosis, BOS

and/or melorheostosis. Twelve patients with melorheostosis were analysed but only one had an identified *LEMD3* mutation; the other 11 appeared to be normal in this gene. However, they described in the paper that this sporadic patient with melorheostosis had osteopoikilosis lesions in the right elbow and shoulder on radiographs which lead us to think that the diagnosis for this patient should not be sporadic melorheostosis. Therefore, no mutation of *LEMD3* was actually identified in their sporadic melorheostosis patients. Our study supplies further evidence that *LEMD3* may not be involved in the cause of sporadic melorheostosis.

There have been many reports of families and individuals with co-occurrence of osteopoikilosis and melorheostosis in the recent literature [409]; [408]; [410];. Whether this fact is a coincidence without implying that *LEMD3* is the disease-causing gene in the latter condition is still not clear. Clinically, melorheostosis is a non-hereditary disorder involving the bones in a segmental pattern, whereas osteopoikilosis is a rather mild disseminated bone disorder inherited as an autosomal dominant trait. In analogy to various autosomal dominant skin disorders for which a type 2 segmental manifestation has been postulated, Happle stated that melorheostosis may be best explained in such co-occurrence as a type 2 segmental manifestation of osteopoikilosis [416].

The *LEMD3* gene codes for a protein of the inner nuclear membrane that inhibits both the BMP and TGF β signalling pathways [335, 417]. Whether any other genetic defect involving this pathway is responsible for melorheostosis needs to be further clarified.

In conclusion, our study supplies further evidence that *LEMD3* mutations may not be the cause of sporadic melorheostosis. The elucidation of the underlying genetic defect in a larger population of melorheostosis patients is undertaken in this group.

4.5 GENETIC STUDIES ON FAMILIAL CHONDROCALCINOSIS

Manuscript in preparation

INVESTIGATING ANKH AND ENPP1 IN SLOVAKIAN FAMILIES WITH CHONDROCALCINOSIS

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Keywords: familial chondrocalcinosis, genetics, FBAT, pyrophosphate

ABSTRACT

Familial articular chondrocalcinosis was first reported in 1963. It is characterized by multiple calcifications of hyaline and fibrous cartilage in the joints and intervertebral disks. Mutations in *ANKH* have been identified in several pedigrees as a monogenic cause for this disorder. ANKH is a key protein in pyrophosphate metabolism since it is responsible for pyrophosphate transport across the cell membrane. The objective of this work was to screen *ANKH* and *ENPP1*, two key genes in pyrophosphate metabolism, in Slovakian kindreds with familial chondrocalcinosis.

DNA samples from 25 individuals (10 affected, 15 unaffected) from 8 families were obtained. The promoter, coding regions and intron-exon boundaries of *ANKH* and *ENPP1* were sequenced.

Twelve DNA sequence variants, six in each gene, were identified. All the variants had been previously identified. None segregated with the disease. Due to the high rate of homozygous parents (affected and unaffected) the genetic informativeness of these families was scarce for association studies. Our results suggest that neither *ANKH* nor *ENPP1* mutations are the cause of chondrocalcinosis in these families, indicating that possibly other major genes are involved in the aetiopathogenesis of this condition. A possible role of variant K121Q of *ENPP1* needs to be further investigated.

INTRODUCTION

Familial articular chondrocalcinosis (CC) was first reported in 1963 by Zitnan and Sitaj [272]. These authors investigated 10 pedigrees with several individuals affected by multiple calcification of the hyaline and fibrous cartilage of joints and intervertebral disks. The main manifestation of this clinical entity was the inflammatory involvement of one or more joints, with episodes lasting 15 days, on average. This was reported to occur as two clinical forms: (1) polyarticular, developing in youth and progressing relatively rapidly, in a large number of joints; and (2) oligoarticular, at a more advanced age, affecting fewer joints, and showing milder symptoms, which soon becomes stationary. The first observations suggested that CC showed familial aggregation. A genetic investigation was performed in 5 families with the polyarticular, oligoarticular forms or both forms. The authors classified this as a hereditary condition; they referred to the difficulty in defining the precise mode of inheritance but proposed the involvement of single gene mutations [272].

Many families with CC, segregating as an autosomal dominant trait, were described thereafter. Two loci were then linked to this disorder. (1) In 1995, Baldwin *et al.* [276] reported genetic linkage to CCAL1 on chr. 8q, in a large family from Maine with early onset CC and severe degenerative osteoarthritis (OA) [CCAL1, MIM 600668]. The disease causing gene was never identified, but there is a strong possibility that it is primarily related to OA and that calcium pyrophosphate deposition (CPPD) is secondary, enhanced by the degenerative changes in cartilage. (2) Another form of autosomal dominant CC, caused by CCAL2 [CCAL2, MIM 118600] was also identified in English families with CPPD. Linkage to chromosome 5p was established [277]. Subsequently, in two other CPPD-CC unrelated families, another locus also on 5p, mapping just centromeric to the first one, was identified through recombination analysis [278]. The identification of the mouse *ank* gene, and demonstration that loss-of-function mutations in it can cause ectopic hydroxyapatite deposition [31], led to postulate that gain-of-function mutations in its human homologue might be responsible for the disease in those families [268]. This was subsequently confirmed in other families with autosomal dominant CPPD disease [279], [32, 280].

The *ANKH* gene maps to human chromosome 5 (5p15.1) and encodes a 492 amino acid multiple-pass transmembrane protein (ANK) which transports the inorganic pyrophosphate (PPi) across the plasma membrane into the extracellular compartment [282]. ANK function is essential in joints to inhibit mineral formation in joints and maintain mobility [418]. Constitutional or joint-specific loss-of-function mutations in *ank* lead to an increase in intracellular PPi and its decrease extracellularly, promoting the excess calcium hydroxyapatite (HA) formation found in the *ank/ank* mouse [31, 418].

There is also evidence suggesting the involvement of the enzyme ectonucleotide pyrophosphatase 1 (ENPP1) in ectopic calcification disorders such as the ossification of the posterior longitudinal ligament (OPLL) of the spine [291] and idiopathic infantile arterial calcification [IIAC, MIM 208000] [87]. The normal activity of this enzyme, a membrane-bound glycoprotein that produces PPi from ATP, inhibits pyrophosphate related calcification and mineralization. *ENPP1* maps to 6q22-q23 and comprises 25 exons that encode a protein expressed in a variety of tissues including matrix vesicles of bone and cartilage.

The “tiptoe walking” (*ttw*) mouse, a natural model for OPLL, is homozygous for a nonsense mutation in *Enpp1*. It exhibits ossification of the spinal ligaments and articular cartilage calcification by hydroxyapatite deposition [86]. Mutations in *ENPP1* cause IIAC, a condition characterised by periarticular and arterial hydroxyapatite deposition, with low activity of ENPP1 and low PPi levels [87]. It would thus be possible that gain-of-function mutations in this gene are involved in the development of CPPD-CC, however preliminary studies did not identify a major effect from common genetic variants of *ENPP1* [292]. A recent study identified two novel mutations in mouse strains with low bone mass: one in *Enpp1* (C397S) and the other in *Ptpn6* (I482F). The phenotype of the *Enpp1* mice showed severe joint disease and widespread vascular calcification. This supports a role for the *Enpp1* in the disease associated with mineralization of articular cartilage and vascular calcification [419].

The main objective of this study was to screen for *ANKH* and *ENPP1* mutations in individuals with familial CC of Slovakian origin by direct sequencing of promoter, exons and intron-exon boundaries to try to find a cause for CC in these pedigrees.

PATIENTS, MATERIALS AND METHODS

A total of 25 individuals (10 affected, 15 unaffected) from 8 Slovakian families were included in the study. Patients were affected with chondrocalcinosis. Individuals from families 6, 7 and 8 are descendants from those initially reported by Zitnan and Sitaj in 1963. The relevant parts of the pedigrees can be seen in Fig.1.

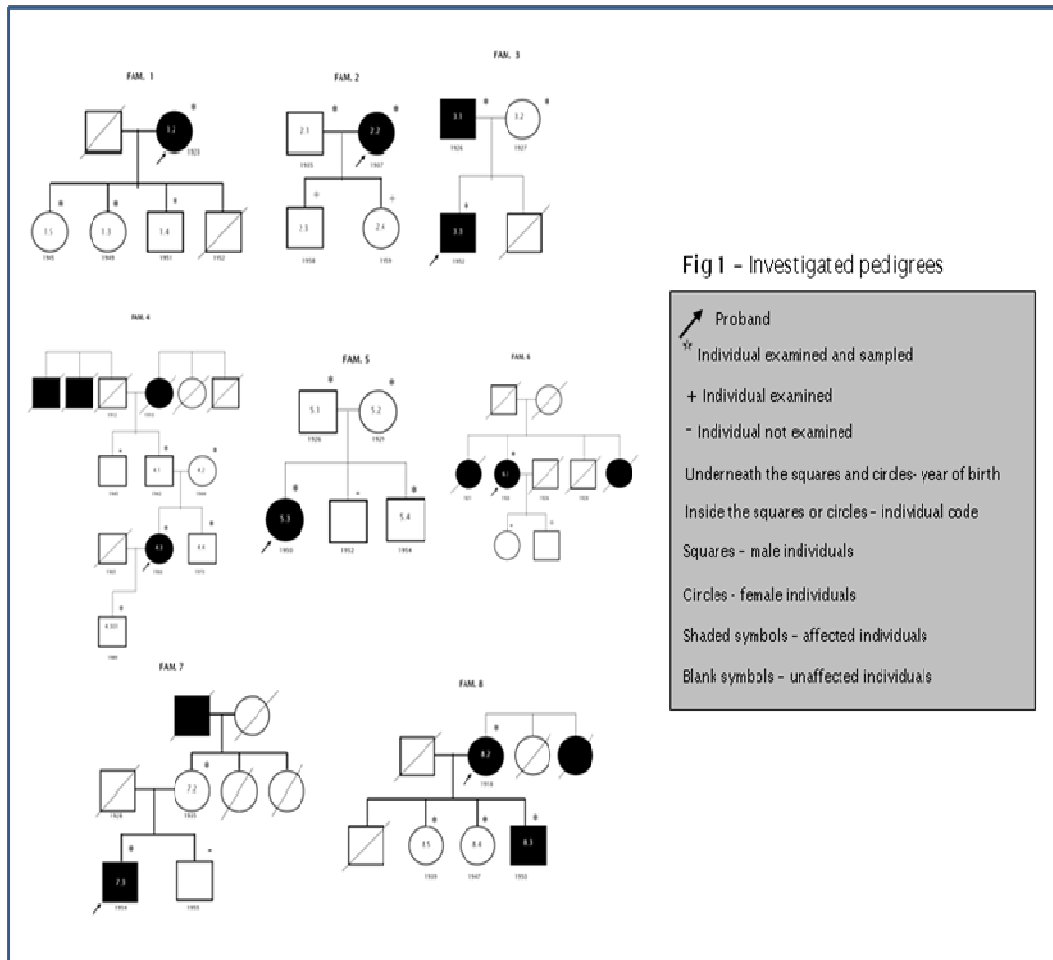


Figure 1- Investigated pedigrees

DNA was extracted by salting out from peripheral blood samples that were stored at -20°C at the Wellcome Trust Centre for Human Genetics (Oxford, UK).

ANKH mutations were assessed by direct sequencing of promoter and coding regions, using primers for flanking intron sequences. 50 µl PCR reactions were

optimized for all primer sets and performed with: (10x) PCR Gold Buffer; 200 μ M of each dNTP; 1, 2 or 3 mM of MgCl; 0.4mM of Primer Mix and 1U of Ampli TaqGold (Applied Biosystems); deionized distilled water was used to attain the final volume. Annealing temperature and magnesium concentration were adjusted until maximum specificity was reached, for each set of primers. PCR amplification was performed under the following conditions: 94°C-14min; 94°C-1min, 50-60°C-1min, 72°C-1min (35 cycles); 72°C – 10 min; 4°C – hold. PCR products were purified in Millipore MANU3050 filter plates on a vacuum manifold. Purified products were resuspended on water. Sequencing reactions of 10 μ l used: 2 μ l of Ready Reaction Premix (2.5x) with BigDye Terminators V.3.1; 2 μ l of BigDye Seq Buffer (5x); 1 μ l of Primer and 5 μ l of purified PCR template; deionized distilled water was used to attain the final volume. Reactions were then subjected to the following conditions: 96°C – 5sec; 96°C – 10sec, 50°C – 5sec, 60°C – 4min (25 cycles). Purification using Corning Glass Fiber Filter Plates followed the protocol: one volume of sequencing reaction was mixed with 2 volumes of 6M potassium iodide and this mixture was then transferred to Corning 0.25mm Glass fiber plate. The wells were filled with 200 μ l of 80% Ethanol (Sigma, Molecular Biology Grade) and centrifuged at 2000xg for 1 minute. Flow through was discarded. Ethanol wash was repeated. The plate was allowed to dry for 1 min. 40 μ l of MilliQ sterile water was added to each well and incubated for 1 minute. Elution of sequencing reactions into a new collection plate was performed by centrifuging (2000xg) for 2 minutes. Sequences were compared using FACTURA and SeqScape (Applied Biosystems).

Mutations in *ENPP1* were also assessed by direct sequencing of promoter and coding regions, using primers located in flanking intron sequences. PCR reactions were optimized for all primer sets and 20 μ l were setup with: (10x) PCR Gold Buffer; 200 μ M of each dNTP; 1, 2 or 3 mM of MgCl; 0.4mM of Primer Mix and 1U of Ampli TaqGold (Applied Biosystems). 100 ng of DNA were added to each reaction. Deionized distilled water was used to attain the final volume. Annealing temperature and magnesium concentration were adjusted until the maximum specificity was reached for each set of primers. Primer set for Exon 1 required the utilization of BSA (3%) and DMSO (10%). PCR amplification was performed using the following conditions: 94°C-14min; 94°C-30sec, 50-60°C-30sec, 72°C-1min (35 cycles); 72°C – 15 min; 4°C – hold. PCR products were purified either in Millipore MANU3050 filter plates, by centrifugation (PCR products resuspended in MilliQ

water), or by ExoSAP-IT (GE Healthcare) according to the manufacturer's instructions. Sequencing reactions of 10µl were setup using: 2 µl of Ready Reaction Premix (2.5x) with BigDye Terminators V.3.1; 2µl of BigDye Seq Buffer (5x); 1µl of Primer and 5µl of Purified PCR Template. Deionized distilled water was used to attain the final volume. Sequencing reactions were then subjected to the following thermal programme: 96°C – 5sec; 96°C – 10sec, 50°C – 5sec, 60°C – 4min (25 cycles). Purification of sequencing reactions using Sephadex G50 Superfine Gel (Amersham) on Millipore Multiscreen Plates (MAHVN4550) following the protocol: 400 µl of Sephadex G50, dissolved in MilliQ water, were loaded onto each well, and centrifuged at 4000rpm for 5 min. Water was discarded and sample was loaded; after centrifuging at 4000rpm for 5 minutes sample was collected on another plate. Sequences were analysed and compared using the software Sequencing Analysis, SeqScape (Applied Biosystems) and BLAST.

To minimize data errors, extensive checking procedures were used. To each DNA variation identified an allele designation was assigned and samples were checked for errors in Mendelian patterns of inheritance using SUPER-LINK PedCheck [420] (available online at: <http://bioinfo.cs.technion.ac.il/superlink-online/makeped/pedcheck.shtml>).

A family-based association test was performed to analyse the association of each of the identified variants with the trait under study – CC. The FBAT (Family Based Association Testing) software, version 2.0.2C [306], which was build on the original TDT method, is based on the distribution of the offspring genotypes conditional on any trait information and on the parental genotypes. If the parental genotypes are not observed, the test statistics is conditioned on the sufficient statistics for the offspring distribution. The expected distribution is derived using Mendel's law of segregation. Biallelic tests were performed using the additive genetic model. This model was used since several studies have shown that the additive model has good power, even when the true model is not an additive one. To increase the power of the test the offset option was used. This option allows both affecteds and unaffecteds to contribute to the test statistics diminishing its variance.

RESULTS

Sequencing revealed 6 sequence variants each in *ANKH* and *ENPP1*. These were named according to the Human Genome Variation Society (HGVS) guidelines and can be seen in table 2. All had been previously described.

Table 2. Sequence variants in *ANKH* and *ENPP1* identified in Slovakian pedigrees with familial CC. Ht stands for heterozygous and Hm for homozygous.

Gene	Location	DNA Sequence variants	Affected Individuals	Unaffected Individuals
<i>ANKH</i>	Promoter	c. -368_-370insGGC	1.2 (Ht)/ 2.2(Ht) /3.3(Ht)/ 4.3(Hm) /6.3(Ht)/ 7.3(Hm) /8.2(Hm) /8.3(Ht)	1.3 (Ht)/ 1.4(Ht)/ 1.5(Ht)/ 2.1(Ht)/ 3.2(Ht)/ 4.1(Ht)/ 4.301(Hm)/ 4.4 (Ht)/ 5.1(Ht)/ 5.2(Ht)/ 5.4(Ht)/ 7.2(Hm)/ 8.4 (Ht)
	Promoter	c. -64_-71delTCGCCCCG	1.2 (Hm)/ 2.2(Hm)/ 3.1(Hm)/ 3.3(Ht)/ 6.3(Ht)/ 8.3(Ht)	1.3 (Ht)/ 1.4(Ht)/ 1.5(Ht)/ 2.1(Ht)/ 3.2(Ht)/ 4.1(Ht)/ 4.4(Ht)/5.2(Ht)/ 8.4(Ht)
	5'UTR	c. -4 G>A	8.3(Ht)	2.1(Ht)/ 5.2(Ht)/ 5.4(Ht)/ 8.5(Ht)
	Exon 2	c. 294 C>T	1.2 (Ht)/ 8.2(Hm)/ 8.3(Ht)	5.1(Ht)/ 5.4(Ht)/ 8.4(Ht)/ 8.5(Ht)
	Exon 8	c. 963 A>G	5.3 (Ht)	5.1 (Ht)
	3' UTR	c.*31 A>G	3.1(Ht)/ 6.3(Ht)/ 8.2(Ht)/ 8.3(Ht)	8.4(Ht)
<i>ENPP1</i>	5' UTR	c. -35 G>T	None	1.3 (Ht)/ 1.5 (Ht)
	5'UTR	c. -32 C>G	4.3 (Ht) / 8.3 (Ht)	4.2(Ht)/ 7.2(Ht)/ 8.4(Ht)
	Intron 2	c. 313+9 G>T	6.3(Ht)	5.4(Ht)
	Exon 4	c. 513 A>C	4.3(Ht)/ 5.3(Hm)/ 6.3(Ht)	4.2 (Ht)/ 5.1 (Ht)/ 5.2(Ht)/ 5.4(Ht)
	Intron 12	c. 1274-8delT	3.1(Ht)/ 5.3(Ht)/ 6.3(Ht)/ 7.3(Ht)/ 8.2(Ht)/ 8.3(Ht)	1.3(Ht)/ 5.1(Ht)/ 5.2(Ht)/ 5.4(Ht)/ 7.2(Ht)/ 8.4(Ht)
	Intron 20	c. 2101-11delT	3.1(Ht)/ 3.3(Ht)/ 5.3(Ht)/ 6.3(Ht)	5.1(Ht)/ 5.2(Ht)

None of the variants found in either gene segregated with the disease or was exclusively found in affected individuals. Allele frequencies in affected and unaffected individuals were compared with a chi-square test. No significant difference was observed.

Results from the FBAT analyses for *ANKH* and *ENPP1* can be seen in table 3.

Table 3. FBAT results, only for informative families, for association of *ANKH* (Chr5; genetic position -15.2) and *ENPP1* (Chr6; genetic position 23.2) mutations with CC using an additive model.

Gene/Physical Position of variant	Marker	Allele	Allelic Frequency	Number of informative families	Z-score	P value
ANKH/14762276	c. -4 G>A	G	0.925	1	-1.000	0.3173

ENPP1/132170834	c. -35 G>T	A	0.075	1	1.000	0.3173
		G	0.911		-1.000	0.3173
		T	0.089		1.000	0.3173
ENPP1/132214061	c. 513 A>C	A	0.836	2	-1.000	0.0833
		C	0.164		1.000	0.0833
ENPP1/132232183	c. 1274-8delT	Wt	0.784	2	-0.392	0.6949
		del	0.216		0.392	0.6949
ENPP1/132245167	c. 2101-11delT	Wt	0.871	1	0.000	1.0000
		del	0.129		0.000	1.0000

As the phenotype under study is dichotomous (affected vs unaffected) other options were available for testing possible association. As variant c.513A>C in *ENPP1* might suggest a weak association to the disease status, FBAT testing was repeated with the option *offset* (-o), optimized by the software, which is used for diseases considered to be common. In this case, the offset value was set to 0.5 meaning that both affected and unaffected individuals contribute equally to the statistics but have opposing signs.

Table 4. Results of FBAT -o analyses performed with additive model, bi-allelic tests

Gene/Physical Position of variant	Marker	Allele	Allelic Frequency	Number of informative families	Z-score	P value
ENPP1/132170834	c. -35 G>T	G	0.911	2	-0.816	0.4142
		T	0.089		0.816	0.4142
ENPP1/132214061	c. 513 A>C	A	0.836	2	-2.449	0.0143
		C	0.164		2.449	0.0143
ENPP1/132232183	c. 1274-8delT	Wt	0.784	3	-0.738	0.4605
		del	0.216		0.738	0.4605
ENPP1/132245167	c. 2101-11delT	Wt	0.871	1	0.000	1.0000
		del	0.129		0.000	1.0000

FBAT analyses showed the c. 513 A>C variant in *ENPP1* to be significantly associated with chondrocalcinosis, when using an additive model (Z score=2.449; p=0.0143).

DISCUSSION

Mode of inheritance

Familial chondrocalcinosis has been reported by many as a dominant disorder, either autosomal or sex-linked, with variable penetrance [315, 421, 422] [318, 423] [359] [361] [253, 424] [425] [363]. In a few studies it was reported as an autosomal recessive [253, 426] whereas other could not establish a model of inheritance [303, 427].

These Slovakian pedigrees (Fig. 1) are not consistent in terms of inheritance and genetic heterogeneity cannot be excluded. Nevertheless, an autosomal dominant (with incomplete penetrance and/or variable expressivity) is perhaps the most likely, if a single mode was to explain all the families; in particular family 3 shows a father-to-son transmission. No parental consanguinity was elicited, but a frequent recessive trait or complex/polygenic inheritance might be implied as well.

Mutations causing CC

This was the first attempt to search for genetic factors in the original families reported with articular chondrocalcinosis. The reduced number of individuals and the short number of generations available lead us to sequence two candidate-genes, *ANKH* and *ENPP1*. These were selected taking into account their role in pyrophosphate homeostasis and mineralization. *ANKH* mutations have been identified in several pedigrees with familial CC. *ENPP1* is the human homologue of a gene producing exuberant articular mineral formation in knockout mice.

According to the Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk>) 5 *ANKH* mutations have been associated with CC (table 4). Six mutations have been linked with craniometaphyseal dysplasia (CMD) [MIM 123000], an autosomal dominant condition characterised by hyperostosis and sclerosis of the skull, together with flaring and abnormal modelling of the metaphyses of long bones [85, 281]. Another mutation (-4bp G-A transition) was found to promote enhanced expression of ANKH protein and excess generation of extracellular inorganic pyrophosphate [428].

Table 4. ANKH mutations associated with CC.

Mutation type	Modification	Aminoacid change	Location	Reference
Regulatory	CGGG(G-A)ACTATG	Non coding	5' UTR, -4bp from initiation codon	[428]
Regulatory	CCCA(C-T)GGCG	Non coding	5'UTR, -11 bp from initiation codon	[32]
Missense	CCG-ACG	Pro-Thr	Codon 5	[280]
Missense	CCG-CTG	Pro-Leu	Codon 5	[279]
Missense	ATG-ACG	Met-Thr	Codon 48	[32]
Missense	GGG-AGG	Gly-Arg	Codon 389	[85, 283]
Small deletion	GAGgagAAT	Glu deletion	Codon 490	[32]

According to the HGMD, there are 4 *ENPP1* mutations associated to myelopathy, in particular with ossification of the posterior longitudinal ligament (OPLL) (table 5) and many more related to IIAC.

Table 5. ENPP1 mutations associated with OPLL.

Mutation type	Codon change	Aminoacid change	Location	Reference
Missense/Nonsense	CTT-CCT	Leu-Pro	Codon 39	[291]
Missense/Nonsense	TCC-TTC	Ser-Phe	Codon 235	[291]
Splicing	T-C	Met-Thr	-14	[429]
Small deletion	ATTtTCG	Non coding Intron 20	-11	[291]

Family-based association studies

Twelve DNA sequence variants, six in each of *ANKH* and *ENPP1*, were identified in 25 individuals. All had been previously reported. None was specific of patients or showed a clear segregation with the disease in any of the pedigrees. Thus, a transmission disequilibrium test (TDT) was performed using the FBAT tool kit. Family based association tests are very helpful in genetic studies since they test for linkage as well as association avoiding false associations caused by admixture in populations. However, the high number of homozygous parents for the variants limited the informativeness of these families [430].

ANKH variants

Two of the *ANKH* variants that we found in these families have been reported in 2003 [431] while studying association of *ANKH* with ankylosing spondylitis: ANKH-TR a triplet repetition GGC located 16bp upstream of TATA box in the 5'UTR of *ANKH* (c. -368_-370insGCC), and ANKH-OR, a 8bp repeat c.-65_-72delTCGCCCCG; alleles with 7 or 8 GGCs were the most common for the triplet repeat whereas the most common alleles for the octanucleotide repeat had 1 or 2 units.

According to *ANKH* reference sequence (UCSC Genome Bioinformatics Site), in which the wild type allele contains 7 GGC repeats and 2 GTCGCCCC repeats, there were several Slovakian individuals with 8 GGC units and only 1 GTCGCCCC repeat; 8 of the 10 affected individuals presented this variation (3 in the homozygous and 5 in the heterozygous form); however, 13 of 15 unaffected individuals also carried it (11 heterozygotes and 2 homozygotes) making it an unlikely disease-associated variant.

It is estimated that the -4bp G>A transition (5'UTR) is ~8 fold more frequent in subjects with sporadic CC than in healthy subjects [428]. We found that 4 unaffected individuals were heterozygous for this mutation whereas only one affected was heterozygous; no homozygotes were found. This study is too small though to identify other than large genetic effects, such as those associated with a monogenic disease-causing mutation.

Polymorphisms in exons 2 (c.294C>T) and 8 (c.963 A>G) are missense changes with no pathological consequence known. Both were investigated in ankylosing spondylitis patients with negative results [432]. These polymorphisms have also not been associated with CC [428].

Mutations in the 3'UTR of genes have been found in several diseases [433-435]. They are known to influence the gene expression due to presence of regulatory elements involved in the control of nuclear transport, polyadenylation, and subcellular targeting as well as rate of translation and degradation of mRNA. Four patients from 3 families had an A>G substitution at position 31 in the 3'UTR. Only one unaffected individual presented this mutation.

ENPP1 variants

A T deletion at position 11 upstream from the splice acceptor site of intron 20 (c.2101-11delT) has been associated with OPLL [291], and with obesity and type II diabetes [436]. Four patients from 3 of our families had this mutation; 2 unaffected parents transmitted it to their affected offspring raising the possibility of incomplete penetrance of a causative mutation in those families. FBAT analysis did not show any significant statistical association between this variant and the disease.

The c.513 A>C variant of *ENPP1* was the only one showing significant association to the disease in our families. It produces the amino acid change K121Q. It was initially described in strong association with insulin resistance, but has been associated with other complex disorders, including obesity [437] and type II diabetes [438] [436].

Previous investigations of a putative role of *ENPP1* in chondrocalcinosis have not found any significant association [292]. However, an association between *ENPP1* polymorphisms and hand osteoarthritis was reported in the Chuvaska population (Russian Federation); the authors suggested an important role of *ENPP1* in idiopathic osteoarthritis [439].

CONCLUSIONS

Neither *ANKH* nor *ENPP1* mutations are the cause of this type of CC in these families. Due to the small number of families and the high rate homozygosity in parents, for the 12 variants identified, the informativeness of TDT in these families was small. Our results suggest a possible role of K121Q but this needs to be further investigated.

5. GENERAL DISCUSSION

5.1 Genetic approaches to study ectopic calcification

Ectopic calcification is the result of a process, which is complex to investigate due to the vast number of direct and indirect physical, biochemical, environmental and genetic factors involved.

The use of large DNA collections of case-controls, animal models, large sets of markers for whole genome wide scans and updated bioinformatics programmes has proven to be a big advantage for the study of genetic factors influencing health and disease. The rapid development of genetic analysis has brought new and interesting insights to the ectopic calcification theme, and is providing evidence of the importance of several genes in the bioregulation of Pi/PPi levels which control mineralization. Major discoveries in this area of knowledge have also been achieved using mouse models [84].

One of the most interesting recent studies on the biomineralization topic was carried out by Murshed et al. [440]. The authors investigated the reason why there is a spatial restriction of ECM in mineralizing bone. In a series of exquisite experiments they showed that:

1) Mineral regulation of bone mineralization is sturdily determined by Pi which is the critical ion in the induction of mineral crystals in a given ECM; its role is strongly antagonized by PPi the presence of which prevents the incorporation of Pi in the nascent hydroxyapatite crystals. The ratio of Pi to PPi is thus determinant in for ECM mineralization, while being largely determined by the TNAP function.

2) Two types of genes are necessary and sufficient for ECM mineralization; one is a reliever of the inhibitor (PPi), exemplified by *TNAP*, and the other encodes the scaffold in which the deposition occurs, exemplified in this study by fibrillar collagen. Surprisingly, neither of these genes are osteoblast specific; however, osteoblasts in bone, odontoblasts in teeth and hypertrophic chondrocytes of the growth plate cartilage are the only cell types that co-express simultaneously these genes.

3) Physiological ECM mineralization is regulated locally, and not systemically, by these two types of genes. The role of Pi and TNAP is conserved between mice and humans, meaning that studies performed in mice models may be informative in regard to the management of ectopic ECM mineralization in humans.

This study has importantly shown that bone mineralization is determined partly by the ability of osteoblasts to remove pyrophosphate, a physiological inhibitor of mineralization, from their surrounding ECM, and by the concomitant presence of a fibrillar collagen-rich network in the bone ECM.

Both bone mineralization and ectopic calcification are influenced by the balance between the expression between genes that act on Pi and P_{PPi} ratio (e.g *ANKH*, *ENPP1* and *TNAP*). In our study, *ANKH* and *ENPP1* were investigated in several kindreds with familial chondrocalcinosis (CC). After direct sequencing of these genes no disease causing mutation was found. Other genes involved in Pi metabolism, such as *TNAP*, are of interest for future studies.

5.2 Genetic studies in families diagnosed with CPDD/DISH

Calcium pyrophosphate deposition disease - CPDD, which is the most common form of chondrocalcinosis, was one of the disorders characterised by ectopic calcification investigated in our study. Twelve Azorean pedigrees were used, as work material, for the identification of the genetic cause of their early-onset and exuberant phenotype.

Clinical and radiological presentation was rather variable among and within these 12 kindreds ranging from asymptomatic to acute (pseudogout) and chronic patterns of presentation. Polyarticular osteoarthritis was identified in a number of patients; a widespread inflammatory polyarthritis mimicking rheumatoid arthritis was identified in one family and more localized arthritis with non-peripheral joint sites including the cervical spine was also seen. The presence of the calcification of the anterior longitudinal ligament (ALL) of the spine, characteristic of diffuse idiopathic skeletal hyperostosis (DISH), another rheumatic disorder, increased the variability of the phenotype. Epidemiological and genetic studies become much more complex under these circumstances. Although stringent criteria for affection status were set for the selection of probands, there were many

difficulties, in practice, in the selection of a group of families with homogeneous phenotypes among them.

It is now widely accepted that Pi and PPi levels are determinant for the development of rheumatic calcific disorders such as CPDD and DISH. A recent study reported the exact Pi:PPi ratio in which normal mineralization is superseded by pathological mineralization [441].

One would assume that cases where ossification of ligaments occur (DISH), the level of PPi would be diminished and hydroxyapatite crystals would be ectopically formed. On the other hand, cases where CPPD crystals were identified in knee joints would suggest the existence of excessive levels of extracellular PPi meaning that the underlying molecular mechanism would favour PPi production. The simultaneous observation in these families, of ossification in certain spinal and peripheral ligaments and the identification of pyrophosphate crystals in synovial fluid is, to say the least, of great interest.

The main questions that, until now, remained to be answered were: (1) “why are these simultaneous mechanisms so prevalent in these individuals?” and (2) “are both these mechanisms the result of a single genetic cause?”. The answers to these questions are still not fully acknowledged.

The simultaneous presence of calcium pyrophosphate and basic calcium phosphate (BCP=hydroxyapatite) in a single joint is not unusual [442, 443]. Previous case reports on individuals with concomitant presence of several crystals include a triple crystal – monosodium urate monohydrate, pyrophosphate dihydrate and basic calcium phosphate – identified in a single joint [444]. There has also been the description of a familial form of destructive hydroxyapatite deposition with arthropathy in which CPPD and BCP coexist - Milwaukee shoulder [445]. The genetic cause for this arthropathy was investigated in one large Italo-Argentinian kindred through linkage studies in several candidate genes, but the results were uninformative. The gene *COL2A1* was sequenced; however, no pathogenic mutations were identified, thus excluding also this gene as a cause of this disorder [322]. No further results were made public from this study.

The molecular mechanisms underlying the simultaneous presence of these crystals remained unclear.

The Azorean pedigrees with CPPD in synovial fluids have not been analysed for basic calcium phosphate crystals; thus, its presence cannot be excluded. Are the different etiological processes occurring simultaneously for a particular reason in these individuals or is one process leading to the other? Previous reports described the high incidence of DISH in patients with pseudogout. An interesting report by Okazaki et al [446] stimulates other investigators to examine pseudogout patients for spinal changes. Are these cases also a coincidence?

It is known that the process of ossification in DISH starts in the innermost layer of the anterior longitudinal ligament, at the site of its attachment to the vertebra, and then extends to meet other arm of ossification coming from the subjacent vertebrae [447]. Authors have suggested that this results from the abnormal osteoblast cell growth or activity in the bony-ligamentous region [447]. Abnormal osteoblast growth and proliferation may be the result of high insulin (encoded by *INS*) and growth hormone levels that have been seen in DISH patients [233 455]. Insulin-like growth factor I (encoded by *IGF-1*) stimulates alkaline phosphatase (ALP) activity and type II collagen expression in osteoblasts. These factors, essential for mineralization, would determine the fate of ligament ossification in DISH.

Matrix-Gla Protein (MGP), a mineralization inhibitor, has been reported to occur in higher levels in DISH patients, when compared with healthy control subjects, and has been suggested as a marker of hyperostosis [239]. This seems a contradiction, since higher levels of a mineralization inhibitor would most probably impair mineralization. The proofs to that comes from *mgp/mgp* mouse model and the observation that loss of function mutations in *MGP* are the genetic cause of the Keutel Syndrome, an autosomal recessive disorder characterised by abnormal cartilage calcification, midfacial hypoplasia and peripheral pulmonary stenosis [448]. It is known that MGP regulates mineralization by binding to HA and that the binding affinity is dependent of numerous factors that include the presence of free calcium, phosphate and magnesium ions. Free calcium increases MGP binding in a dose-dependent manner whereas phosphate and magnesium decreases it in a dose-dependent and independent manner, respectively [449].

The link between DISH and CPDD may then emerge from the MGP function. Studies on the role of MGP performed in bovine endothelial cells have shown that, among other things, there was a dose dependent expression increase in the

expression of vascular-endothelial-growth factor-A (VEGF-A). To determine the mechanism by which human MGP (hMGP) increases VEGF-A, the authors performed transfection assays to evaluate the effect of MGP on transforming growth factor- β 1 (TGF- β 1) and bone morphogenetic protein 2 (BMP2). The results showed a strong enhancement of the activity of TGF- β 1 and inhibition of BMP2 [450].

The specific role of TGF- β 1 in CPDD has been investigated by Derfus et al. These authors concluded that TGF- β is capable of increasing the capacity of articular chondrocyte-derived MV to generate PPI via nucleoside triphosphate pyrophosphohydrolases (NTPPPH) and precipitate calcium in the form of CPPD mineral. These data support the concept that this growth factor is a key role in cartilage matrix CPPD deposition [451]. BMP2 share the ability of TGF- β to induce PPI release [452].

The hypothesis that we put forward to explain the co-occurrence of both crystals in the same individuals is the following: genetic variations that increase the expression of *IGF-1* would lead to the stimulation of ALP activity and type II collagen expression promoting the ligament ossification in DISH, as previously suggested [447]. It is known that IGF-1 decreases chondrocyte expression of *MGP* transcripts [453]. The lower expression of *MGP* would, in turn, favour the expression of BMP2, which would significantly stimulate the PPI-generating enzyme NTPPPH, without altering the specific activity of PPI-degrading enzyme ALP [454]. This would lead to a major increase in PPI release and its deposition as CPPD crystals. However, this would suggest that DISH patients should have an increased frequency of CPPD chondrocalcinosis. There is some data combining the positive correlation of these diseases [446].

Several patients from the Azorean pedigrees have CPPD crystals in their knee joints. Although highly improbable, this could also be the result of several metabolic conditions as previously discussed. These conditions can lead to either a reduction of the breakdown of PPI or to an increase of its production, by several different mechanisms.

The reduction of the PPI breakdown, which is performed by the enzyme alkaline phosphatase (ALP) it may occur for several reasons which include reduced levels of the enzyme itself, the presence of its inhibitors or a deficiency of the cofactors.

As the affected individuals had normal serum ALP, not showing any signs of hypophosphatasia, enzyme level was surely not the cause for the CPDD. The excess of alkaline phosphatase inhibitors such as calcium, was not seen in these individuals. Deficiency of ALP cofactors such as magnesium (hypomagnesemia) was also not identified in any of the affected or unaffected individuals. However, the presence of excess iron, which can work either as an inhibitor of ALP or as an enhancer of crystal nucleation, was seen in several individuals. Still, it is not probable that this was the cause of the phenotypes identified in these families, as not all the individuals with iron excess had radiological anomalies, and many of those who showed them did not have excessive serum iron. Yet, its influence on disease severity cannot be excluded.

Results from the investigation of the Azorean pedigrees suggested that although this appears to be an autosomal dominant condition with major action of a single gene, there is probably locus heterogeneity among the families studied. This would mean that mutations in several different genes could be accounting for a similar phenotype. Other linkage studies using only samples from families with suggestive linkage would be impracticable since the number of individuals would be too small and the power of the study would be highly reduced. Considering the results of the linkage analysis in these pedigrees, in which there was not a single chromosomal area positively and indisputably linked to the disease, it is possible that the phenotypes are not the result of mutations in a single gene, though it cannot also exclude it.

5.3 Paleopathological study

The access to the skeleton of one of the individuals investigated in this study was an excellent opportunity to examine a number of details that would not be possible any other way.

There are many overlaps between radiological changes in AS, DISH and CPDD. Very often is difficult to establish a definitive diagnosis among this group of conditions. Enthesopathic calcifications and ossifications are major manifestations of all three disorders and differential diagnosis may be complex. Due to these similarities, many have postulate that, at least some, susceptibility

factors involved in the aetiopathogenesis of these disorders should be shared among them.

The genetics of AS, in particular, evolved very rapidly; linkage studies revealed a considerable number of genes, outside the MHC region, where it lies the most important susceptibility factor known, and HLA specific allele [169, 455]. HLA-B*27 has been for years the strongest association reported to AS. Linkage analysis has been quite valuable for the understanding of this spondyloarthropathy; some genes such as *KIR*, *IL-23R*, and *ERAP1* have been consistently associated with AS in several studies [456-458]. Further genome-wide studies in large sets of case-control cohorts will likely improve the knowledge over this disorder [459].

Several investigations reported CPDD families with phenotypes that can be confounded with AS or with DISH. Of particular relevance are the reports of Reginato in the Chiloe Islands from Chile [427], van der Korst *et al.* in Holland [315], and the Azorean pedigrees investigated in this thesis [368]. It is interesting to notice that patients from Argentina are the descendents of the islanders that in the 16th century admixed with Portuguese and Spanish, which came from Iberian Peninsula.

The Terceira island, in the Azores, where familial CPDD seems to be an endemic manifestation, was a secure port for travelling ships, and was populated with people from different parts of Europe, namely an important group of Flemish. It is thus possible that the descriptions in these three papers may be associated with migrations of people across continents with the Azores as a common path. All these reported forms may show an axial phenotypic expression very similar to AS, and are frequently diagnosed in a subset of early-onset familial CPDD.

The differential diagnosis between DISH and AS has been the subject of discussion in multiple publications. A majority of authors emphasize the similarities of the spinal radiographic features between both diseases. DISH criteria were set to allow the differential diagnosis. The absence of ankylosis, erosion, sclerosis, or intra-articular osseous fusion of apophyseal joint, is essential for the diagnosis of DISH. Another criterion helping in the differential diagnosis between DISH and AS is the sacroiliac disease, always present in AS patients on their middle ages; however, it is important to state that sacroiliac

abnormalities may be found also in DISH, although erosions and intra-articular bony ankylosis are rarely present.

Although it is clear that recent research revealed different molecular mechanisms underlying the pathogenesis of these conditions, the molecular basis of the enthesopathic / ossification phenomena which is a common finding of AS, CPDD and DISH, is not known yet. Our study was important for the reason that it added another evidence of possible genetic links between disorders which are normally thought to be very diverse. Future research will address this issue and may possibly clarify the reason why some radiographic features of these diseases are quite similar.

5.4 Identification of Osteopoikilosis causing mutation in *LEMD3*

A rare autosomal dominant bone disorder was diagnosed in one of the Azorean pedigrees under investigation. Osteopoikilosis is frequently an incidental finding with no clinical consequence [400]. Albers-Schönberg reported it in 1915 describing this disorder as a rare structural anomaly of the skeleton [401]. In 1916, Ledoux-Lebard et al. introduced the term “*osteópoecilie*”, originating from Greek and meaning “spotted bones” [402]. Other denominations of this clinical entity include “disseminated osteopathy” [403] and osteosclerosis disseminata familiaris [404].

Clinical characteristics previously reported were confirmed in this study; i.e. this disorder was identified in both men and women from that kindred, with an autosomal dominant pattern of transmission, including male-to-male transmission.

A novel mutation in exon 8, of gene *LEMD3*, was identified in the heterozygous state in the pedigree. This mutation, a C to T substitution, at position 2032bp (cDNA), changed amino acid at 678 from Arginine to a Stop codon, and segregated with the phenotype of all affected individuals. None of the unaffected individuals in this Azorean pedigree, or any of the healthy controls studied, had this mutation.

5.5 Current genetic approaches to study CPDD/DISH families

The success of mapping human genes related to diseases, when working on a family based strategy, depends greatly on the number of informative meioses analysed. As a result, the small size of most pedigrees used in genetic studies diminishes the genetic resolution that can be obtained.

This study started with a relatively high number of full clinically characterized pedigrees to investigate. A whole genome wide linkage study was performed in twelve of those pedigrees. Pedigrees for linkage study were selected in order to obtain, in terms of phenotype, the most homogeneous group as possible. They were all collected in Terceira island which is one of the nine islands of the Azores archipelago. This island has a surface area of approximately 382Km² and 59000 inhabitants.

Most of the pedigrees, however, were small, and few meioses were available. A whole genome linkage study was performed with 327 microsatellite markers. Linkage analysis did not reveal the exact chromosomal region associated with this disorder but a particular region in chromosome 16 was identified as an interesting area for further studies. This region is a relatively large area of approximately 15cM. Chromosome 16 has a total number of 1270 genes; the chromosomal area with suggestive linkage, 16q12.1-16q22.1, comprehends 252 genes several of which are candidates for a role in this disorder.

Duplication of this exact same zone has been associated with spina bifida in a 7-year-old girl. An abnormal karyotype was identified involving the long arm of chromosome 11 and fluorescent in situ hybridization (FISH) to metaphase chromosomes revealed an insertion of part of chromosome 16 (16q12.1-q22.1) on chromosome 11[391].

Interesting genes in region 16q12.1-q22.1 include:

1. *HAS3*: (16q22.1): encodes the hyaluronan synthase 3, a membrane-bound enzyme which is involved in the synthesis of the unbranched glycosaminoglycan hyaluronan, or hyaluronic acid, a major constituent of the extracellular matrix. Interestingly, there are numerous reports associating the use of intra-articular hyaluronan with the development of CPPD arthritis [392-394];

2. A cluster of cadherin genes which encode calcium-dependent adhesive proteins that mediate cell-to-cell interaction and are involved in the structural and functional organization of cells in various tissues [395].
3. *SLC12A3*, which is quite important in ion homeostasis, and mutations of which can cause Gitelman Syndrome (GS) [OMIM 263800]. GS is an autosomal recessive form of familial hypokalaemia and hypomagnesaemia [396]. There are many studies reporting the familiar association of GS with CC due to CPPD deposition [397, 398]; this is thought to be resultant from chronic hypomagnesaemia [396].

Unfortunately, parametric and NPL could not provide sufficient evidence of linkage. However, NPL linkage analysis gave significantly better results. Important factors for success of parametric analysis include the correct assumption of the disease model and adequate selection of molecular markers. Besides genetic heterogeneity, the cause for the failure of linkage analysis can also be related to the number of markers used (not sufficient) or the large distance between markers.

Even if the pedigrees investigated in this study were unrelated, which is clearly unexpected, due to small number of inhabitants in the island and the high number of HLA alleles they share, they could certainly share ancestral chromosomal segments. Taking advantage of this fact it is possible to locate *identical by descent* (IBD) markers inherited from a common ancestor locating in this way a possible genetic region of interest.

An investigation of IBD markers in 10 individuals from these kindreds is currently being performed.

5.6 Genetic studies on familial chondrocalcinosis

Many candidate genes have been and are currently being investigated in the expectation to supply innovative and significant data for the development of future novel therapeutic targets.

In this study we have investigated two strong candidate genes for familial CPDD. *ANKH*, a transmembrane P_{pi} transporter, which is until now the only known

monogenic cause for familial CC; and *ENPP1*, a nucleotide pyrophosphatase, mutations in which have been shown to produce a phenotype resembling CC.

Mutations were screened after direct sequencing of the promoter region, exons and intro-exons boundaries of both genes. The objective was to find patient-specific mutations which would suggest that one of these candidates is the actual gene involved. This is the most popular method since it is easily applicable and comparatively rapid. None of the mutations identified in the 24 individuals studied, however, was patient-specific.

Mutations identified in these individuals were tested for association with the disease using the software package FBAT (Family based association tests). Tests were uninformative for two main reasons: 1) the number of individuals studied was very small; 2) the mutated allele frequencies were too low being the large majority of imputed haplotypes being homozygous for the wildtype allele.

A new set of candidate genes will be selected for direct sequencing. Among them, genes with direct influence in phosphate homeostasis should be considered for analysis in these families e.g. *IGF-1*, *MGP*, *FGF23*, *OPN*, and *klotho*.

5.7 Treatment options for rheumatic ectopic calcification disorders

Treating these disorders has been the main objective of the previously mentioned studies. Identifying an efficient treatment for DISH, CPDD, AS and related spondylarthropathies, would be a great success for many investigators who spent many years and immeasurable human and monetary resources working on this subject and very important for their patients and families.

Several therapeutic target suggestions have been suggested in recent years. In AS treatment, tumour necrosis factor (TNF) blockers do not prevent fusion so it is crucial to understand the ankylosis process to develop ways of preventing this. For AS, as well as for genetically related disorders such as inflammatory bowel disease and psoriasis, the TH17 system is presented at the front line for a possible new treatment option [460]. As for CPPD and DISH simple analgesic or nonsteroidal antiinflammatory drugs are usually the only prescribed treatment, although colchicine and methotrexate have been prescribed with some success in

CPDD [461, 462]. None of these symptomatic treatments will actually lead to a regression of the disease. It remains to be seen if, in future years, an efficient treatment for this problem is to be found.

5.8 Future perspectives

An already ongoing study in the Azorean families includes the use of SNP chips trying to identify common chromosomal areas, shared only by affected individuals. This strategy can also be applied to Slovakian kindreds since there is no need of having large sets of individuals to be successful.

In case this strategy fails to identify any chromosomal region associated with the disease, a larger cohort of individuals affected by this disease, as well as matched controls, will have to be identified and characterised for another approach.

To overcome the difficulties of identifying the chromosomal area(s) where the gene(s) responsible for this particular disease is located, a genome wide association study (GWAS) can ultimately be performed.

GWAS can be an extremely powerful approach to identify the genetic basis of different human diseases, being especially valuable for complex diseases. It is becoming increasingly more popular and valuable due to the progress of the HapMap project and the accessibility of dense genotyping chips [463]. In order to increase the power of the marker sets under use, methods have been proposed to predict individual genotypes at untyped loci from other marker sets, through imputation [464].

For the statistical approach to data interpretation various imputation algorithms have been used. Four publicly available programs for genotype imputation (BEAGLE, IMPUTE, MACH and PLINK) were recently evaluated in terms of genotype prediction and imputation efficacy. BEAGLE, IMPUTE and MACH were almost identical in imputation accuracy and efficacy whereas PLINK performed consistently poorer [464].

Online catalogues of SNPs have recently been created, based on published genome-wide association studies, for use in investigating genomic characteristics of trait/disease-associated SNPs (TASs) (<http://www.genome.gov/gwastudies/>)

[465] and as a tool for investigators to select the gene or region of interest and the best SNPs based on GWAS results, linkage disequilibrium (LD) and predicted functional characteristics of both coding and non-coding SNPs (<http://www.niehs.nih.gov/snpinfo>) [466]. Online resources, together with the most appropriate bioinformatic tools, are becoming indispensable for future investigations of the role of common variants in complex disease etiology [465]. Although precious in some studies, there are a number of limitations along with association studies. The main limitation of this method includes the huge number of cases needed for investigating complex disorders. This number increases as the effect of the gene variant decreases. The choice of the control group in any association study is also absolutely crucial and rather complex. Population stratification and admixture may yield false-positive results and should be strongly avoided. However, performing such a study in Terceira island would probably have as the major confounder the common ancestral origin if this is both associated with gene frequency and is an independent risk factor for the disease (*i.e.* has an influence on disease risks conditional of genotype) [467].

5.9 Concluding remarks

Investigating the genetic cause of disorders characterised by ectopic calcifications proved to be rather complex. Our study was the starting point of an investigation that can contribute decisively for the better understanding of this fascinating theme.

The results of this study indicate that a single strategy will not be sufficient for the identification of the genetic basis of this disorder characterised by ectopic calcification. A whole genome wide linkage study was performed in twelve severely affected kindreds; this strategy was not satisfactory in terms of results as no significant susceptibility *loci* were identified. Another strategy, an association study, is currently being performed and results will soon be available.

Although not presenting all final answers for the numerous questions posed initially, there were some major achievements coming out from this thesis:

1. The gathering and organisation of first clinical, radiological, biochemical and genetic data of several Azorean families suffering from an early-onset

disorder mainly characterised by enthesopathic calcifications. This information will be precious for the establishment of a more homogenous group of families, the investigation of which will continue in more detail in the future.

2. Based on the clinical and genetic results obtained in this study, stringent criteria for definition of phenotype will be set. Accurate phenotype definition is essential to study the genetic background of any disease. The number of families will be increased; several other families are currently being identified and investigated. Defining family-specific phenotypes can actually help to organize the observed clinical heterogeneity, increasing the chances of finding chromosomal regions containing susceptibility genes.
3. A particular region in chromosome 16 was identified as an interesting area for further studies in this disorder. Both parametric and NPL could not provide sufficient evidence of linkage; however, NPL linkage analysis gave significantly better results.
4. Since the families diagnosed with familial CC were too small for a linkage approach, candidate genes were chosen for direct sequencing analysis. Several nucleotide changes, as well as small deletions and insertions, were identified in both affected and unaffected individuals. None of the variants was patient-specific. The results suggest that neither of the investigated genes, including *ANKH* which is the only cause for monogenic CC known, is the cause for the disorder in these families. Future studies will screen other possible candidate genes and hopefully other monogenic cause for CC may be identified.
5. The identification of a novel mutation in *LEMD3* segregating with osteopoikilosis was another important positive finding from this investigation. Although this disorder is mainly asymptomatic, the identification of novel mutations associated with the disease will contribute for a better knowledge of the action of this protein.

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7. ANNEXES – PUBLISHED PAPERS

All the published papers related to the theme, referred in pages 4 and 5, will be presented in annexe according to the following list:

Annexe 1

Bruges-Armas J, **Couto AR**, Timms A, Santos MR, Bettencourt BF, Peixoto MJ, Colquhoun K, McNally EG, Carneiro V, Herrero-Beaumont G, Brown MA. Ectopic calcification among families in the Azores: clinical and radiologic manifestations in families with diffuse idiopathic skeletal hyperostosis and chondrocalcinosis. *Arthritis Rheum.* 2006 Apr;54(4):1340-9.

Annexe 2

Couto AR, Brown MA. Genetic factors in the pathogenesis of CPPD crystal deposition disease. *Curr Rheumatol Rep.* 2007 Jun;9(3):231-6.

Annexe 3

Couto AR, Bruges-Armas J, Peach CA, Chapman K, Brown MA, Wordsworth BP, Zhang Y. A novel LEMD3 mutation common to patients with osteopoikilosis with and without melorheostosis. *Calcif Tissue Int.* 2007 Aug;81(2):81-4.

Annexe 4

Bruges Armas J, **Couto AR**, Bettencourt BF. Spondyloarthritis, Diffuse Idiopathic Skeletal Hyperostosis and Chondrocalcinosis. In: Molecular Mechanisms of Spondyloarthropathies. Series: Advances in Experimental Medicine and Biology, Vol. 649; López-Larrea, Carlos; Díaz-Peña, Roberto (Eds.) 2009, Approx. 340 p., Hardcover ISBN: 978-1-4419-0297-9

Annexe 5

Jordana X, Galtés I, **Couto AR**, Gales L, Damas M, Lima M, Bruges-Armas J. The coexistence of ankylosing spondylitis and diffuse idiopathic skeletal hyperostosis - a postmortem diagnosis. *Clin Rheumatol.* 2009 Mar;28(3):353-6.

ANNEXE 1

Ectopic Calcification Among Families in The Azores

Clinical and Radiologic Manifestations in Families With Diffuse Idiopathic Skeletal Hyperostosis and Chondrocalcinosis

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Bruno Filipe Bettencourt,¹ Maria José Peixoto,¹ Katherine Colquhoun,³ Eugene G. McNally,³
Victor Carneiro,⁴ Gabriel Herrero-Beaumont,⁵ and Matthew A. Brown²

Objective. Twelve families that were multiply affected with diffuse idiopathic skeletal hyperostosis (DISH) and/or chondrocalcinosis, were identified on the island of Terceira, The Azores, potentially supporting the hypothesis that the 2 disorders share common etiopathogenic factors. The present study was undertaken to investigate this hypothesis.

Methods. One hundred three individuals from 12 unrelated families were assessed. Probands were identified from patients attending the Rheumatic Diseases Clinic, Hospital de Santo Espírito, in The Azores. Family members were assessed by rheumatologists and radiologists. Radiographs of all family members were obtained, including radiographs of the dorsolumbar spine, pelvis, knees, elbows, and wrists, and all cases were screened for known features of chondrocalcinosis.

Results. Ectopic calcifications were identified in 70 patients. The most frequent symptoms or findings were as follows: axial pain, elbow, knee and metacarpophalangeal (MCP) joint pain, swelling, and/or deformity, and radiographic enthesopathic changes. Elbow and

MCP joint periarticular calcifications were observed in 35 and 5 patients, respectively, and chondrocalcinosis was identified in 12 patients. Fifteen patients had sacroiliac disease (ankylosis or sclerosis) on computed tomography scans. Fifty-two patients could be classified as having definite (17%), probable (26%), or possible (31%) DISH. Concomitant DISH and chondrocalcinosis 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. The mean age at which symptoms developed was 38 years.

Conclusion. These families may represent a familial type of pyrophosphate arthropathy with a phenotype that includes peripheral and axial enthesopathic calcifications. The concurrence of DISH and chondrocalcinosis suggests a shared pathogenic mechanism in the 2 conditions.

Diffuse idiopathic skeletal hyperostosis (DISH) (ankylosing hyperostosis, Forestier-Rotes Querol's disease) (MIM no. 106400) is a common condition among the elderly. It is characterized by ossification of the anterior longitudinal ligament, affecting in particular the right side of the spine, with preservation of the intervertebral disc space. While spinal involvement in DISH is nearly universal, in some cases peripheral entheses may also be involved (1). It has been reported that ochronosis, hypophosphatasia, acromegaly, hemochromatosis, hypomagnesemia, fluorosis, and hypervitaminosis A may also cause similar enthesopathy (2).

DISH occurs more frequently in men, and its prevalence increases with age, affecting mainly subjects >40 years of age (3). Its prevalence may be as high as 25% in men and 15% in women >50 years of age (4).

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However, accurate measures of disease prevalence are not well established, since large-scale radiographic studies would be required because of the high prevalence of asymptomatic disease.

There have been few systematic studies of the clinical consequences of DISH (5). One of the first reported studies on this condition showed that stiffness and mild middle-to-low back pain were found in 80% of patients, dysphagia was present in 28%, elbow pain in 13%, and heel problems in 23% (6). Peripheral nerve entrapment has also been described in conjunction with DISH (7).

It has been reported that 50% of DISH cases are complicated by a similar condition, ossification of the posterior lateral ligament (OPLL) (MIM no. 602475) (8), suggesting that they share common etiopathogenic factors. OPLL is mostly found in Asian populations, in particular among the Japanese, where it has been reported to have a prevalence of 1.9–4.3% (9). The etiology of DISH is unknown, with pathogenic roles of degenerative, inflammatory, and metabolic factors (in particular, diabetes mellitus) having been suggested (3,10).

Chondrocalcinosis, a common cause of joint pain and arthritis, particularly in the elderly, is characterized by the deposition of calcium-containing crystals in articular cartilage, synovial membranes, and, less often, in periarticular soft tissues (11,12). The physicochemical mechanisms that cause this deposition are still unknown, but a key role for disordered inorganic pyrophosphate (PPi) metabolism is suspected (13). The disease is most commonly seen in a sporadic form in elderly people, with a prevalence of 25% in the population >85 years and 3% in people ages 65–69 years (14). Chondrocalcinosis is often associated with endocrine and metabolic diseases such as hemochromatosis and hypophosphatasia, among others (15). A hereditary form of “chondrocalcinosis articularis” was first reported by Zitnan and Sitaj (16) in 7 Czechoslovakian kindreds in 1963. Since then, many reports of familial aggregation have been reported in different ethnic groups, most of them showing an autosomal-dominant inheritance (17–23). In contrast, very few cases of familial DISH have been reported (24–27), and to our knowledge, there is only one description of DISH in association with calcium pyrophosphate dihydrate (CPPD) crystals (8).

Reports of familial chondrocalcinosis and shoulder arthropathy suggest that disordered PPi metabolism may play a role in calcific rotator cuff diseases (28). It has also been suggested that diffuse spinal ligamentous

calcification could be mediated through increased PPi generation by ligament fibroblasts encasing the spine (29). Although many factors are likely to be involved in the regulation of calcification and ossification processes, studies of chondrocalcinosis and DISH have implicated control over extracellular PPi levels as one of the most important factors in their etiopathogenesis (13).

The Azores islands are located in the middle of the Atlantic Ocean, and were very important in the 15th and 16th century sea traffic between Europe and America. The islands were officially populated in 1439. European colonization of the archipelago was surprisingly swift, since all the current main towns already existed by 1550. Portuguese documents show that the islands were populated after 1432 by Portuguese, Dutch (Flemish), Spanish, Berber, Jewish, and Italian immigrants. In addition, black African slaves were brought to the islands. Presently, the Azores archipelago is an autonomous Portuguese region with ~250,000 inhabitants (30).

Herein we report the clinical, radiologic, and some genetic features of families from Terceira Island in The Azores who have ectopic calcifications. These families have disease characterized by enthesopathic musculoskeletal calcifications, frequently fulfilling the radiologic criteria for DISH, and which, in some cases, are associated with CPPD chondrocalcinosis.

PATIENTS AND METHODS

Twelve cases of severe early-onset disease were identified. Three individuals with sacroiliac (SI) disease and severe enthesopathic spinal disease were identified from the European Prospective Osteoporosis Study radiologic cohort (31).

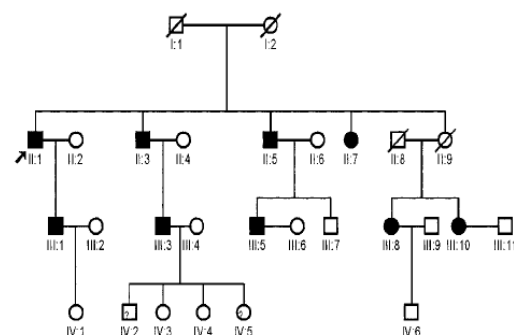


Figure 1. Pedigree showing a pattern of transmission compatible with an autosomal-dominant monogenic disease. Solid symbols = affected subjects; open symbols = unaffected subjects; solid symbol with arrow = proband; question marks = possibly affected.

Table 1. Ectopic calcifications in 70 individuals from 12 families*

Family member/sex	Age at symptom onset, years	Current age, years	Joint involvement by radiography							Other diseases	CPPD crystals	
			Knees	Hands	Wrists	Elbows	Cervical spine	Dorsal spine	Lumbar spine			Hips
Family 1												
1/F	ASY	42	—	—	—	+	+	—	—	—	—	—
2/F	32	42	—	—	—	+	+	—	—	—	Osteopoikilosis, nephrolithiasis	—
3/M	40	74	+	+	—	+	+	+	+	—	Osteopoikilosis	—
4/M	ASY	46	+	—	—	+	+	—	—	—	Osteopoikilosis, nephrolithiasis	—
Family 2												
1/M	42	64	—	+	+	+	+	—	+	—	Nephrolithiasis	+
2/M	41	53	—	+	—	+	+	—	—	—	Nephrolithiasis	—
3/M	50	60	—	+	—	+	+	—	—	—	—	—
4/M	50	73	—	+	+	+	—	—	+	—	Rheumatoid arthritis?	—
5/M	55	82	+	+	+	+	—	—	+	+	—	—
6/F	42	46	+	—	—	+	+	+	+	—	Shoulder calcification	—
7/F	45	59	+	+	—	+	+	+	+	—	Nephrolithiasis	+
8/M	22	57	+	+	—	+	—	+	+	—	Nephrolithiasis, Osgood-Schlatter disease	—
9/M	18	79	+	+	+	+	+	+	+	+	Nephrolithiasis	—
10/M	40	75	—	+	+	+	†	†	†	—	Congenital scoliosis	—
11/M	21	31	+	—	—	—	—	—	—	—	Osgood-Schlatter disease	—
Family 3												
1/F	40	72	+	+	—	+	—	+	+	—	—	—
2/M	30	76	+	+	+	+	+	—	+	—	Shoulder osteophytosis	+
3/F	20	73	+	—	+	+	—	+	+	—	—	—
4/M	ASY	55	—	—	—	+	+	—	—	—	—	—
5/F	49	49	—	—	—	—	—	—	+	—	—	—
Family 4												
1/M	35	58	—	—	—	+	+	+	+	—	Bilateral iliac sclerosis	—
2/F	15	40	—	—	—	+	—	—	—	—	—	—
3/F	22	60	—	—	—	+	—	+	—	—	Trochanteric calcification	+
4/F	ASY	63	+	—	—	—	—	—	—	—	—	—
5/M	32	34	—	—	—	—	—	—	—	—	Bilateral superior iliac sclerosis	—
Family 5												
1/M	42	43	+	—	—	+	+	—	—	—	—	—
2/M	ASY	48	+	—	—	+	—	—	—	—	—	—
3/M	35	46	+	—	—	+	+	—	—	—	Nephrolithiasis	—
4/F	60	72	+	+	—	+	+	—	—	+	—	—
5/F	60	71	—	+	—	+	—	+	—	—	—	—
6/F	40	50	—	—	—	—	+	—	+	—	—	—
Family 6												
1/M	60	84	—	—	—	+	+	—	+	—	—	—
2/F	32	50	—	—	—	+	+	—	—	—	Bilateral SI sclerosis	—
3/M	50	86	—	—	—	—	+	+	+	—	Bilateral SI ankylosis	—
4/M	ASY	45	—	—	—	—	—	—	—	—	Calcification iliolumbar ligament	—
Family 7												
1/F	22	48	+	—	—	+	—	+	+	—	—	—
2/M	40	68	+	+	—	+	—	—	+	+	Bilateral superior SI ankylosis	—
3/M	30	36	—	—	—	+	—	—	—	—	—	—
4/F	40	72	+	+	—	+	—	+	+	—	Pseudogout, nephrolithiasis, trochanteric calcification	+
5/M	33	34	—	—	—	—	—	—	—	—	Unilateral superior SI ankylosis	—

The remaining 9 individuals were identified through a record review conducted at the Rheumatic Diseases Clinic of Hospital of Santo Espírito, Angra do Heroísmo, Azores. Probands and family members were interviewed and examined for evidence of arthritis by qualified rheumatologists (JB-A, MAB).

Radiographs of the axial spine, pelvis, knees, wrists, hands, and elbows were obtained from all of the study partic-

ipants (91 individuals). Other appendicular joints were radiographed if there were signs or symptoms of disease, and pelvic computed tomography (CT) scans performed in cases of radiographic SI disease. All radiographs were examined by rheumatologists (JB-A, MAB) and radiologists (KC, EGM). Affected status was determined radiographically, with the diagnosis of DISH being made according to the Utsinger

Table 1. (Cont'd)

Family member/sex	Age at symptom onset, years	Current age, years	Joint involvement by radiography								Other diseases	CPPD crystals
			Knees	Hands	Wrists	Elbows	Cervical spine	Dorsal spine	Lumbar spine	Hips		
Family 8												
1/M	50	81	+	—	—	+	—	+	—	—	Unilateral superior SI ankylosis	—
2/F	50	73	+	+	—	+	—	—	+	—	—	—
3/M	66	71	—	+	—	+	—	—	+	—	—	—
4/F	60	74	+	—	+	+	—	—	—	—	—	—
5/M	27	49	+	—	—	+	—	—	—	—	Pseudogout	—
6/M	40	53	—	—	—	+	—	—	—	—	—	—
7/F	25	55	—	+	—	+	—	+	—	—	—	—
Family 9												
1/F	40	65	+	—	—	+	+	+	+	—	Shoulder calcification + nephrolithiasis	+
2/F	50	69	+	+	—	+	+	+	—	—	Rheumatoid arthritis?	—
3/F	20	63	+	—	—	—	+	—	—	—	—	—
4/F	25	40	—	—	—	—	—	—	+	—	Nephrolithiasis	—
5/F	30	60	+	—	—	—	—	+	—	—	—	—
6/F	35	42	—	—	—	+	+	—	—	—	—	—
Family 10												
1/M	20	50	+	+	—	—	+	+	+	—	Unilateral SI ankylosis + bilateral ankle ankylosis	—
2/M	ASY	49	+	—	—	+	+	+	—	—	—	—
3/M	ASY	36	—	—	—	+	+	—	—	—	—	—
Family 11												
1/M	ASY	44	—	—	—	+	—	—	+	—	—	—
2/M	30	83	+	+	—	+	+	+	+	+	Unilateral SI ankylosis + pseudogout	+
3/M	40	42	—	—	—	+	—	—	+	—	Bilateral superior SI ankylosis (bone bridging)	—
4/M	42	50	+	—	—	+	—	—	—	—	Bilateral superior SI ankylosis (bone bridging)	+
5/M	45	75	+	+	—	+	+	+	+	+	Bilateral SI ankylosis + pseudogout	+
6/M	20	87	+	—	—	+	—	+	+	+	Bilateral superior SI ankylosis + heterotopic hip calcification	—
7/F	20	80	+	+	—	+	+	+	+	—	—	—
8/F	50	59	+	—	—	+	—	+	—	—	Unilateral SI ankylosis + shoulder calcification	—
9/F	40	64	+	—	—	+	—	+	—	—	—	—
Family 12												
1/M	60	83	—	+	—	+	+	—	+	—	—	—
2/M	50	73	+	+	+	+	+	—	—	+	—	—
3/M	40	69	+	—	—	+	—	+	+	—	Shoulder calcification + nephrolithiasis	+
4/F	17	49	—	—	—	+	+	—	—	—	Bilateral iliac sclerosis + nephrolithiasis	—
5/F	30	47	+	—	—	+	+	—	—	—	—	—

* CPPD = calcium pyrophosphate dihydrate; ASY = asymptomatic; SI = sacroiliac.

† Spine affected by scoliosis.

criteria (3). The diagnosis of chondrocalcinosis was based on the classic radiologic evidence of deposition of calcium in the fibrocartilage.

All individuals were screened for secondary causes of chondrocalcinosis, using appropriate biochemical tests: levels of serum calcium, iron, magnesium, phosphorus, glucose, uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, ferritin, transferrin, lactate dehydrogenase, cholesterol, triglycerides, urea, fucosyltransferase (FT3 and FT4), thyroid-stimulating hormone, human growth hormone, and parathyroid hormone were all

measured. *HFE*, *TFR2*, and *FPN1* gene mutations were detected using the Haemochromatosis Strip Assay according to the recommendations of the manufacturer (ViennaLab, Vienna, Austria). Microsatellite marker studies were carried out in order to exclude already known chromosomal areas linked to familial chondrocalcinosis.

Synovial fluid was collected from the majority ($n = 12$) of the individuals who developed acute arthritis symptoms or chronic effusions. All samples were collected from knee joints and were examined by two of the authors (GH-B, VC) under compensated polarized light microscopy to check for the

Table 2. Affected joints in 70 patients with ectopic calcifications*

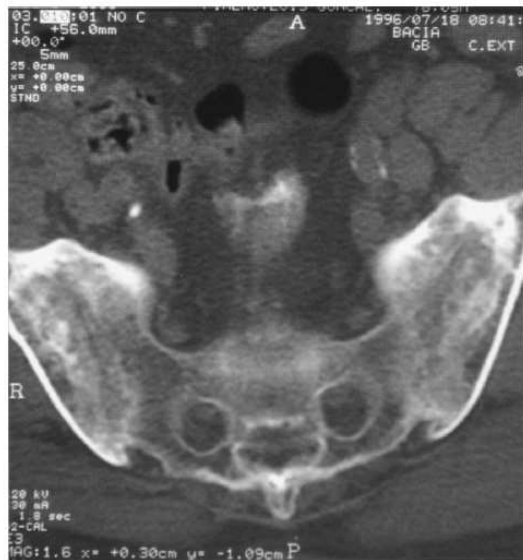
	Radiographic evidence	Pain/swelling	Deformity/limitation
Hands	26 (37.1)	28 (40)	17 (24.3)
Knees	39 (55.7)	36 (51.4)	8 (11.4)
Elbows	58 (82.9)	41 (58.6)	34 (48.6)
Wrists	9 (12.9)	16 (22.9)	3 (4.3)
Spine	57 (81.4)	57 (81.4)	—
Sacroiliac	15 (21.4)	—	—
Hips	8 (11.4)	8 (11.4)	8 (11.4)

* Values are the number (%).

presence of CPPD crystals. The presence of other crystals was not investigated; thus, their occurrence cannot be excluded (32). Selected subjects with SI involvement were genotyped for HLA-B27 (33).

RESULTS

The extended families of all 12 probands were identified, and those living in the Azores were invited to participate ($n = 115$ individuals). Of these, 103 individuals (70 men, 33 women) were studied in entirety. Radiographs were not obtained in 10 young asymptomatic individuals; family members without complete clinical, biochemical, or radiologic assessment were also excluded (2 individuals).

**Figure 2.** Radiograph showing bilateral sacroiliac fusion.

The mean $\pm$ SD pedigree size was 8.6 ± 3.3 (range 5–17). Five pedigrees consisted of 2 generations, and the remaining 7 pedigrees had 3 generations; 3–11 affected individuals were identified in each family. Observation of pedigrees showed a pattern of transmission compatible with an autosomal-dominant monogenic disease (Figure 1). No generation was skipped, and $\sim 50\%$ of descendants of an affected parent were similarly affected, irrespective of parent or offspring sex. Eight probands were born in the same village.

Ectopic calcifications were identified by radiograph in 70 patients (67%; 40 men and 30 women), with a mean age of 59.3 ± 15.1 years (range 31–87) (Table 1). Of these, 9 patients were asymptomatic (mean age 47.6 ± 7.8 years; range 36–63). The mean age at which symptoms developed was 37.8 ± 12.9 years (range 15–66); only 7 patients (10%) developed symptoms after

**Figure 3.** Radiograph of the lumbar spine, showing large flowing osteophytes.



Figure 4. Radiograph showing exuberant osteophytes and articular elbow calcifications.

50 years of age, and 15 patients (21%) were symptomatic before 30 years of age. Two patients, 1 female and 1 male, had previous clinical, serologic, and radiologic diagnoses of rheumatoid arthritis, but enthesopathic calcifications were also identified in the peripheral or axial skeleton. Three members of family 1 (2 men and 1 woman) had radiologic diagnoses of osteopoikilosis (MIM no. 166700); 2 members of family 2 were diagnosed with bilateral Osgood-Schlatter disease, and 13 members of 6 families reported previous nephrolithiasis, including 1 family with 5 affected members. Pseudogout occurred in 4 patients.

Of the 52 patients identified according to the Utsinger criteria (74%; 29 men and 23 women), 12 could be classified as having definite (17%; 7 men and 5 women), 18 as having probable (26%; 9 men and 9 women), and 22 as having possible (31%; 13 men and 9 women) DISH. Twelve patients (17%; 8 men and 4 women) had calcification of knee fibrocartilage and/or calcification of the triangular ligament of the wrist, and 14 (20%) had radiologic findings insufficient for a

definite diagnosis. A diagnosis of concomitant DISH and chondrocalcinosis was made in 12 patients.

Lumbar, dorsal, and/or cervical pain were the most frequent axial symptoms (Table 2). Back pain was "inflammatory-like" in several patients; pain and stiffness were worse during the night or in the morning, improved with movement, and worsened again at the end of the day. The meaning of this symptom was not clear, and we do not know if there is a relationship with the SI disease identified in 15 patients (21%). Only some of these patients had inflammatory-like lumbar pain, and in younger patients with inflammatory-like axial pain, SI disease was not identified on CT scans. Bilateral or unilateral SI ankylosis was identified on radiographs and confirmed by CT scan in 11 patients, and bilateral sclerosis (ilium and/or sacrum) was confirmed in another 4 patients. In 2 patients with early fusion, bridging syndesmophytes could be seen anterior to the SI joint, with no evidence of joint erosion on CT scan. In established ankylosis, cortical remnants of the SI joints, having not been eroded as part

of the disease process, were visible in some cases (Figure 2).

Spinal radiographic abnormalities were identified in 57 patients (83%), and ranged from exuberant osteophytes and syndesmophyte-like spurs (Figure 3) to enthesopathic calcifications. These calcifications were identified as continuous or discontinuous smooth or massive calcifications along the anterior and/or lateral aspect of vertebral bodies.

Elbows, knees, and metacarpophalangeal (MCP) joints were the most frequently affected peripheral joints (Table 2), and symptoms usually started in the same decade as back pain. Strikingly, elbow pain (59%) or deformity (49%) was identified in all families, and elbow pain was often the initial manifestation of disease. Periarticular, capsular, or synovial calcification (50%), and ulnar insertion calcification of the triceps tendon (47%) were the most frequent findings, and were frequently bilateral. These calcifications were usually more exuberant with increasing age of patients, and in some cases, calcification and bone fragmentation were striking, making the limits of elbow structures impossible to define (Figure 4).

Knee disease was associated with pain and/or swelling (51%), and deformity and/or limitation of movement (11%). Superior or inferior patella ligament calcification (29%) and cartilage calcification (16%) were the most frequent radiologic findings. The current mean ($\pm$ SD) age of individuals with knee calcification was 74 ± 8.1 years. CPPD crystals were identified from knee effusions in 10 patients from 8 different families. MCP joints, mainly the second and third, were frequently painful (40%) or deformed (24%), and radiographs showed varying degrees of subluxation of metacarpal heads, sclerosis, narrowing of joint space, periarticular calcifications, and metacarpal head osteophytosis (37%). Radiocarpal compartment disease was identified in 9 patients (13%). Eight patients had triangular ligament calcification, and another patient had bilateral exuberant hyperostosis of the radial extremity. Hip disease was identified in 7 men and 1 woman. Only 1 patient (with definitive DISH) had undergone surgery (20 years before), and radiographs now showed extensive heterotopic ossification with restrictive hip motion.

Biochemical tests revealed normal values of serum calcium, iron, phosphorus, magnesium, potassium, uric acid, AST, ALT, and alkaline phosphatase. Ten male individuals (11%) had ferritin values $>300 \mu\text{g/liter}$ (mean 596, range 333–1,200). Five had transferrin saturation (TS) $>44\%$, and only 3 had *HFE* mutations. One individual was a compound heterozygote (C282Y/

H63D), another was H63D homozygote, and the other was H63D heterozygote. Only 1 woman had a ferritin value $>200 \mu\text{g/liter}$. No mutation was found in this case, and the TS value was normal. All patients had repeated measurements of serum ferritin which confirmed the high values first identified. These levels were not associated with the erythrocyte sedimentation rate or with the disease activity.

Thirty-three individuals from 11 families (32%; 19 men and 14 women) had no ectopic calcifications. Three had pseudogout. One of the patients with pseudogout, a woman age 36 years, had bilateral knee effusion, and CPPD crystals were identified in the synovial fluid.

Linkage to chromosome 5p15 (CCAL2) and chromosome 8p (CCAL1) was excluded by microsatellite marker studies. Further genetic studies are currently being performed.

DISCUSSION

It is not yet known if ectopic calcification occurs with the same prevalence in the other islands of The Azores archipelago. However, on Terceira Island, this condition may possibly represent an endemic and common condition. We are currently investigating several other families with a similar disorder found scattered across the island. Since 8 probands were born in the same village, there is a possibility of a genetic founder effect.

Periarticular calcifications were observed on elbow and MCP joint radiographs in a large group of patients. Furthermore, calcification of knee fibrocartilage and/or triangular wrist ligament was observed in 17% of patients, and pseudogout episodes and CPPD crystals were identified in the majority of the families investigated. Although we have not investigated for the presence of other crystals, the identification of CPPD crystals in all synovial fluids examined suggests that these families have a form of autosomal-dominant CPPD deposition disease (OMIM no. 118600); however, unlike previous reports of this condition, in these families the chondrocalcinosis was complicated in all cases by peripheral and axial enthesopathic calcifications.

It is also possible that the phenotypes observed in these families may correspond to the association of 2 common diseases in this population, DISH and pyrophosphate arthropathy. Familial DISH has previously been reported (24,26), and it is known that if this condition coexists with rheumatoid arthritis or gout, more exuberant new bone formation is observed (34).

In some cases we could not exclude the possibility that the chondrocalcinosis may have developed as a complication of hemochromatosis, since 10 of 91 individuals had high ferritin and TS levels. However, it is unlikely that this could be responsible for more than a small proportion of cases, since other individuals had normal iron stores. Previous studies have demonstrated the rarity of hemochromatosis in this island population (35). Furthermore, the clinical and radiologic features seen in ectopic calcification are, in many aspects, different from the arthropathy of hemochromatosis (15).

Despite the numerous reports of familial chondrocalcinosis since its first description by Zitnan and Sitaj (16), the prevalence of the familial form of chondrocalcinosis is still unknown. Studies of late-onset chondrocalcinosis suggest that the condition has low familiarity (36). During the 2 years of investigation on this island, we came across several other cases with similar phenotypes (data not shown) in which familial aggregation could not be investigated because family members had died or emigrated. Previous reports of family studies on chondrocalcinosis mainly describe 2 different clinical phenotypes: either early onset with polyarticular involvement and variable severity of the arthropathy (OMIM no. 600668) or a phenotype characterized by late-onset oligoarticular chondrocalcinosis with arthritis resembling sporadic disease (37,38). The early-onset phenotype was observed in all the Azorean families described in this study, with initial symptoms starting in the third to fourth decades of life in the majority of the family members.

Classic descriptions of ankylosing hyperostosis of the spine and chondrocalcinosis (6) draw attention to distinctive radiographic abnormalities. Axial skeleton calcification, mainly of the anterior longitudinal ligament, has usually been ascribed to DISH, and is more frequent in the anterolateral dorsal spine. However, calcification of spinal ligaments can also occur as a complication of CPPD deposition disease. In spinal chondrocalcinosis with CPPD, the initial site of crystal deposition appears to be the outer fibers of the anulus fibrosus, which may produce opaque linear or tumoral densities that resemble the syndesmophytes of ankylosing spondylitis (AS) (39). Furthermore, widespread involvement of the anulus and the nucleus pulposus may occur, especially in patients with familial disease, producing extensive disc calcification. While most of our patients had no radiographic disc abnormalities, disc calcification was observed in some. Whether disseminated CPPD deposition caused the spinal disease in our patients is the subject of current postmortem studies.

Previous reports have highlighted the similarities in cases of spinal CPPD, DISH, and AS (17,39,40), and it can be difficult in some patients to differentiate these conditions. This may be one of the explanations for the higher proportion of HLA-B27-negative AS that has been reported in some populations (41). Some of the patients reported here have previously been diagnosed as having seronegative AS, and even with retrospective investigation, we could not be sure of the underlying diagnosis in 1 case. These findings suggest that a proportion of B27-negative cases of AS may represent an ossifying enthesopathy distinct from the immune-mediated process involved in AS.

Sacroiliac bilateral or unilateral complete ankylosis was identified in 11 patients, and bilateral sclerosis (ilium and/or sacrum) in another 4. The first description of SI disease in DISH concludes that capsular ossification is frequent, even early in the disease, but complete bridging of the joint is not reached before the sixth decade (42). Durbach et al (43) described asymmetric superior intraarticular partial fusion, osteophytes with or without bridging, and vacuum phenomenon, while Weisz and Green (44) reported progressive obliteration of the SI joints in 4 of 11 patients with longstanding Forestier's disease. DISH primarily affects the superior and ligamentous area, leaving unaffected the lower two-thirds of the SI joint, which is a synovial component in the normal SI joint. Sclerosis or erosions of the SI joint have been described in chondrocalcinosis (45), and calcification in the articular cartilage of both the ilium and sacrum was noted in a postmortem examination study (46). Thus, both DISH and chondrocalcinosis can cause SI disease, and both may be relevant in the SI ankylosis or sclerosis identified in our patients.

CPPD deposition is thought to be a consequence primarily of raised extracellular PPI levels, which are known to reduce the formation of hydroxyapatite crystals. The frequent coexistence of both crystal types in synovial fluid samples from patients with pseudogout has not yet been properly explained. Identification of the genetic mechanism operating in these families may provide insights into this apparent conundrum.

Osteopoikilosis is an autosomal-dominant skeletal dysplasia that has been described in association with several conditions. It is characterized by the presence of multiple hyperostotic areas in the metaphyses and epiphyses of the long bones, carpus, tarsus, and membranous bone (47). The association of osteopoikilosis with DISH or chondrocalcinosis in 1 of the families described herein has not, to our knowledge, been reported previously; the relevance of this finding is not

known but warrants further investigation. A loss-of-function mutation in *LEMD3* (MIM no. 607844), a gene that is involved in bone morphogenetic protein and transforming growth factor β signaling, coding for an integral protein of the inner nuclear membrane, has recently been identified (48).

The multiple clinical and radiologic manifestations in these Azorean families support the presence of a common etiopathogenic pathway involved in pyrophosphate arthropathy and DISH. Identifying the genetic causation of this disorder is likely to add to our currently limited understanding of the etiopathogenesis of these common disorders of ectopic calcification.

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ANNEXE 2

Genetic Factors in the Pathogenesis of CPPD Crystal Deposition Disease

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Crystal deposition is a very complex process ruled by numerous factors. A small but important proportion of cases of chondrocalcinosis are monogenic, and many of the genes involved have been identified. These genetic findings strongly point to control of the level of extracellular inorganic pyrophosphate as the primary mechanism for their association with either calcium pyrophosphate dihydrate or hydroxyapatite deposition. However, effects on extracellular inorganic pyrophosphate levels do not explain the mechanism of association in all of these monogenic diseases. Further, there are likely to be several as yet unidentified genes that are important in this common condition. This review highlights what genetic studies have demonstrated about the processes involved in these diverse but related disorders.

Introduction

Mineral deposition is a tightly controlled process in terms of both tissue specificity and rate. The key organelles in this process are chondrocyte and osteoblast matrix vesicles (MVs), in which the principal calcium/phosphate crystals, consisting primarily of calcium hydroxyapatite (HA) or calcium pyrophosphate dihydrate (CPPD), are formed. Inorganic pyrophosphate (PPi) antagonizes the formation of HA crystals; hence the importance of having a tight balance between the levels of extracellular phosphate (Pi) and PPi for normal mineral deposition [1,2]. Factors controlling the rate and tissue specificity of mineralization have been extensively investigated and include factors affecting transport and metabolism of PPi, factors affecting crystal nucleation, and components of the extracellular matrix such as the presence of fibrillary collagen [3••].

Much is known about the control of mineralization through genetic studies, particularly through identifica-

tion of genes involved in monogenic forms of disorders of ectopic mineralization. Mutations in several of these genes affect the rate of generation, transport, and degradation of PPi and have taught us much about the mechanisms controlling mineralization (Fig. 1) [2]. This knowledge has been particularly helpful in characterization of CPPD crystal deposition diseases (CPPD-CDD) for which no animal models currently exist and in vitro studies are of uncertain relevance. This review describes some of those lessons learned from the dissection of genetic causes of ectopic mineralization.

Genetic Epidemiology of Calcium Pyrophosphate Dihydrate Crystal Deposition Disease

Although most cases of CPPD-CDD are nonfamilial, many kindreds with a familial form of this disorder have been reported, indicating a significant genetic impact in the disease. Since the first report of a familial form of CPPD-CDD by Zitnan and Sitaj [4], designated as chondrocalcinosis articularis, many other reports of familial aggregation have been reported scattered throughout the world [5–15,16•]. Two main different clinical phenotypes have been observed in all these kindreds. The first is characterized by the early onset, polyarticular involvement, and variable severity of arthropathy; the second is similar to that observed in sporadic disease cases and is characterized by late-onset oligoarticular chondrocalcinosis (CC) with arthritis [13]. All these reports are characterized by the strong heterogeneity of phenotypes within and between families, and in most cases, by autosomal dominant transmission of disease.

Only a few studies have been conducted to determine the prevalence of CC in first-degree relatives of patients with apparently sporadic CC or pyrophosphate arthropathy. Recurrence risk rates of 11% to 38% have been reported in first-degree relatives [11,17–19]. However, these studies have several limitations, including the small number and mode of selection of index cases, the elevated nonparticipation rate, and most importantly, the absence of a community control population. Only one study has been published comparing the frequency of CC in siblings of cases with that in a control population [20]. This study showed an increase in the unadjusted recurrence

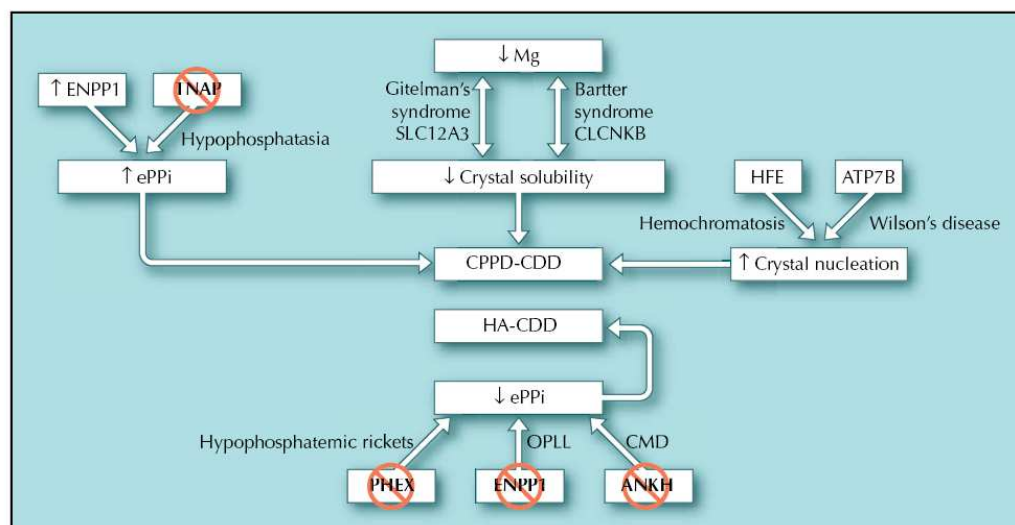


Figure 1. Mechanisms underlying associations of genetic diseases with ectopic mineral deposition. CMD—craniometaphyseal dysplasia; CPPD-CDD—calcium pyrophosphate dihydrate crystal deposition disease; ENPP1—enzyme ectonucleotide pyrophosphatase; ePPi—extracellular inorganic pyrophosphate; HA-CDD—hydroxyapatite crystal deposition disease; Mg—magnesium; OPLL—ossification of the posterior longitudinal ligament; TNAP—tissue-nonspecific alkaline phosphatase.

rate in siblings compared with the general population (odds ratio [OR] = 2.01, 95% CI, 1.13–3.56), but after adjustment for age, body mass index, gender, knee pain, and knee osteoarthritis (OA), this became nonsignificant. It is unclear as to why the adjustment for knee pain and OA was made, and therefore the familiarity of late-onset CC remains uncertain. This is not unusual in late-onset diseases, where genetic effects do not easily translate into increased familial recurrence because of incomplete penetrance. Given the number of monogenic causes of CC, genetic variation will likely affect susceptibility to the disease in later onset, “sporadic” forms.

Monogenic Forms of Chondrocalcinosis ANKH

CPPD CC segregating as an autosomal dominant trait has been described in several families. Two chromosomal regions have been linked to CPPD CC. In 1995, Baldwin et al. [21] reported genetic linkage between markers on human chromosome 8q and the disease in a large family from Maine with early-onset CPPD and severe degenerative OA (CCAL1, MIM 600668). The disease-causing gene has yet to be mapped, but there is a strong possibility that it is primarily related with OA and that the CPPD deposition is secondary, enhanced by the degenerative changes in cartilage.

Greater success has been experienced with a second form of autosomal dominant CPPD deposition disease CCAL2 (CCAL2, MIM 118600). This condition was initially

described and genetically localized in five English families with CPPD crystal deposition. Linkage was established to chromosome 5p, with a multipoint logarithm of the odds score of 4.6 [22]. Subsequently, in two CPPD-CDD unrelated families, one from Argentina and the other from France, another zone in chromosome 5p, mapping just centromeric to the first, was further identified through recombination analysis [23]. The identification of the *ank* gene and demonstration that loss-of-function mutations in the gene cause ectopic HA deposition in the *ank* mouse model [24] led investigators to postulate that gain-of-function mutations in the human homologue of this gene may be responsible for the disease in these families [25]. Subsequently, this finding has been confirmed in other families with autosomal dominant CPPD-CDD [26–28].

Recently, Zhang et al. [29••] demonstrated that a common promoter region polymorphism is associated with sporadic cases of CC. The polymorphism identified in this study, a -4 base pair G-to-A transition, was found in 4% of a 128-patient cohort with widespread premature CC and/or pseudogout [29••]. Thus, *ANKH* variants play a role in both familial and severe forms of sporadic CC.

Mutations in *ANKH* have also been shown to cause another autosomal dominant condition characterized by hyperostosis and sclerosis of the skull, together with flaring and abnormal modeling of the metaphyses of long bones: Jackson’s craniometaphyseal dysplasia (CMD, MIM 123000) [30,31]. As discussed below, this disease has many similarities with the *ank* mouse model.

The *ANKH* gene encodes a 492 amino acid multiple-pass transmembrane protein, which is thought to be involved in the transport of PPi across plasma membrane to the extracellular compartment. Loss-of-function mutations in *ank* lead to an increase in intracellular PPi and a decrease in extracellular PPi, promoting the excess HA formation found in the *anklank* mouse [24].

The exact mechanism by which *ANKH* mutations cause these diseases is unknown. The structure of the ANK protein has not been resolved, probably because of technical difficulties involved in structural studies of transmembrane proteins. Strong evidence has been presented that ANK is a PPi transmembrane transporter. The strongest evidence for this has been for HA-deposition-associated mutations causing either the *ank* mouse phenotype or craniometaphyseal dysplasia. Here, transfection studies and studies in transgenic mice have shown that these *ank* variants are associated with markedly reduced PPi transmembrane transport [32••]. With CPPD-associated mutations, the same group did not observe any significant difference in PPi transport compared with wild-type *ank* [32••]. Transient transfection studies in chondrocyte cell lines have suggested that not all CPPD CC mutations lead to increased extracellular PPi, and some have other effects on chondrocyte maturation and mineralization independent of effects on PPi [29••]. The P5L mutation, associated with CPPD CC, increases type X collagen expression in both transient and stable transfection studies [29••,33•], suggesting an effect of the mutation on chondrocyte maturation and perhaps explaining the association of this mutation with CC.

Part of the difficulty in determining the function of ANK and the effects of *ANKH* mutations may relate to the technically challenging nature of measurement of extra- and intracellular PPi and the fact that available chondrocyte cell lines are not very close matches to primary cells. Further, ATDC5 chondrocytic cell lines express high levels of alkaline phosphatase, which breaks down PPi, and CH-8 chondrocytic cells express high levels of tumor necrosis factor, which has been shown to inhibit PPi elaboration by chondrocytes [34]. Primary chondrocytes dedifferentiate rapidly in culture, complicating their use. The absence of a murine model of CPPD CC is a particular weakness. Even hypophosphatasia mice with severe chronic elevations of extracellular PPi do not develop CPPD CC, perhaps due to the relatively short life span of mice compared with humans or to some characteristic of murine cartilage. The development of such a mouse model would greatly advance our ability to investigate the etiopathogenesis of this disease.

Several other roles for ANKH have been suggested by genetic studies. Besides cartilage, *ank* is widely expressed in the brain, particularly in the thalamus, midbrain, and spinal cord, and its expression is influenced by seizure activity. One family with autosomal dominant CPPD CC

due to *ANKH* mutations is also characterized by a seizure disorder, and the gene has also been investigated as being a possible candidate for idiopathic seizure disorders [35•]. *ANKH* polymorphisms have been reported to influence a variety of skeletal size parameters [36•] and osteoprotegerin levels [37•], but these findings are not robust and await confirmation.

The association of *ANKH* with ankylosing spondylitis (AS) is also rather controversial. The *ank* mouse was initially considered as a potential model of human AS. Further, reports of a clinical association between AS and another disorder of ectopic HA deposition, ossification of the posterior longitudinal ligament (OPLL), raised the possibility that *ANKH* may play a role in the pathogenesis of spinal ossification in AS. A study by Tsui et al. [38] reported a modest association of *ANKH* to AS susceptibility, but research from Timms et al. [39] showed no evidence of association of *ANKH* polymorphisms with either susceptibility to or severity of disease. Additional studies will be necessary to elucidate this subject.

Nucleotide pyrophosphate phosphodiesterase

Genetic evidence suggests the involvement of the enzyme ectonucleotide pyrophosphatase 1 (ENPP1) in ectopic calcification disorders such as the OPLL of the spine [40] and arterial calcification [41]. The normal activity of this enzyme, a membrane-bound glycoprotein that produces PPi from ATP, inhibits pyrophosphate-related calcification and mineralization. The *ENPP1* gene maps to chromosome 6 (6q22-q23) and comprises 25 exons that encode a protein expressed in a variety of tissues including MVs of bone and cartilage. The mouse model of OPLL, "tiptoe walking" (*ttu*) mouse, results from a naturally occurring nonsense mutation in the gene encoding ENPP1 (*Enpp1*^{-/-}). It exhibits ossification of the spinal ligaments and articular cartilage calcification by HA deposition [42]. Mutations in *ENPP1* cause idiopathic infantile arterial calcification, a condition characterized by periarticular and arterial HA deposition, with low activity of ENPP1 and low PPi levels [43]. Thus gain-of-function mutations in this gene may be involved in the development of CPPD-CDD, though preliminary studies do not identify a major effect on CPPD risk from common genetic variants of *ENPP1*, as described below [44•].

Tissue-nonspecific alkaline phosphatase

Tissue-nonspecific alkaline phosphatase (TNAP) is an isozyme of a family of four homologous human alkaline phosphatase genes. It is present in the MVs and has the ability of hydrolyze PPi. In humans, this enzyme is encoded by the gene *TNAP*, which maps in chromosome 1 and contains 12 exons. Deactivating mutations in the *TNAP* gene cause hypophosphatasia (MIM 241500 [infantile form], 146300 [adult type]), characterized by poorly mineralized cartilage and bones, spontaneous bone fractures, CPPD CC, and elevated concentrations of PPi [45].

Table 1. Monogenic diseases complicated by ectopic mineralization

Disease	MIM	Gene	Predominant crystal type	Postulated mechanism
Cranio metaphyseal dysplasia	123000	<i>ANKH</i>	Hydroxyapatite	Loss of function of ANKH, leading to decreased extracellular PPI
Hypophosphatemic rickets (X-linked)	307800	<i>PHEX</i>	Hydroxyapatite	Low Pi, unknown PPI
Hemochromatosis (classical)	232500	<i>HFE</i>	CPPD	Enhanced crystal nucleation
Wilson's disease	277900	<i>ATP7B</i>	CPPD	Enhanced crystal nucleation
Gitelman's syndrome	263800	<i>SLC12A3</i>	CPPD	Magnesium deficiency, causing functional hypophosphatasia, decreased crystal solubility
Bartter syndrome (classical)	607364	<i>CLCNKB</i>	CPPD	Magnesium deficiency, causing functional hypophosphatasia, decreased crystal solubility
Hypophosphatasia	146300 (adult), 241500 (infantile)	<i>TNAP</i>	CPPD	High PPI

CPPD—calcium pyrophosphate dihydrate; Pi—phosphate; PPI—inorganic pyrophosphate.

Using double knockout mice, lacking both *TNAP* and *PC-1 (ENPP1)* genes, Hesse et al. [46] showed evidence that *TNAP* and *ENPP1* are key regulators of bone mineralization, suggesting that these genes are antagonistic regulators in this process. To evaluate the hypothetical role of *ENPP1* and *TNAP* in CPPD-CDD, Zhang et al. [44•] screened variants of *ENPP1* and *TNAP* in 128 white patients with sporadic CPPD CC and in 600 healthy controls. No significant difference was observed in the alleles or genotype frequencies between patients and controls, suggesting that these genes are not major genetic determinants of sporadic CPPD susceptibility in whites [44•].

Several other monogenic diseases are complicated by CPPD CC, and in most cases, the disease mechanism appears to be through effects on PPI levels (Table 1). Probably the most common disease association of CPPD CC is hemochromatosis, but the mechanism of this association is poorly understood. Opposite effects of iron salts on CPPD crystal formation have been reported by different investigators, but it is generally thought that they promote crystal nucleation and/or growth [47,48]. It has also been suggested that iron may inhibit pyrophosphatases [49]. Clearly, much needs to be resolved in this particular field.

Hypophosphatemic rickets is another fascinating condition, in which either ectopic HA or CPPD crystal deposition occurs. This condition is caused by mutations in *PHEX*, which cause failure to digest fibroblast growth factor (FGF)-23 (phosphatonin), leading to phosphaturia. The consequences of the phosphaturia are complex and include rickets, growth retardation, and relative deficiency of 1,25(OH)₂D. Although physiologic skeletal mineralization is impaired, thought to be due to the low Pi levels, ectopic HA mineralization can be very problematic, causing spinal stenosis and widespread calcific enthesitis. The

cause of this result is unclear, but may be due to low PPI levels permitting extraskeletal mineralization. If this is the case, then long-term phosphate treatment, even in adulthood, could help prevent this complication of the condition. CPPD CC can also occur. The authors hypothesize that this may be due to high calcium and phosphate levels produced by treatment with phosphate and vitamin D. The use of this treatment to prevent deformity and improve growth is well accepted; however, whether to continue the treatment once skeletal maturity has been reached is unclear, given significant toxicities and other difficulties with the available treatments. Demonstration of a benefit for treatment in patients with widespread calcific enthesitis would support a role for treatment in this subset of patients at least.

Studies of mouse model *Klotho*, which ages prematurely and has osteoporosis and atherosclerosis, have provided fascinating insights into the role of FGF-23 and Pi in these traits [50••]. *Klotho* protein was demonstrated to convert FGF receptor-1(IIIc) into a specific receptor for FGF-23, and in *Klotho* mice this action is lost, leading to loss of FGF-23 function and hyperphosphatemia. Whether the *Klotho* phenotype is due to direct effects of *Klotho* or FGF-23 or due to the mineral imbalance that occurs in these mice (hypercalcemia, hyperphosphatemia) is uncertain. Further studies to assess the role of *Klotho* variants in humans are clearly indicated.

Chondrocalcinosis and Diffuse Idiopathic Skeletal Hyperostosis

A recent investigation by Bruges-Armas et al. [16•] in Azores, Portugal, identified a series of families characterized by both CPPD CC and florid ligamentous calcification,

consistent with the condition diffuse idiopathic skeletal hyperostosis (DISH, MIM 106400). The identification of CPPD crystals in all synovial fluids suggests that these families have a form of autosomal dominant CPPD-CDD; however, unlike previous reports of this condition, in these families, the CC was in all cases complicated by peripheral and axial enthesopathic calcifications. Linkage to chromosomes 5p15 (CCAL2) and chromosome 8p (CCAL1) was excluded by microsatellite marker studies. Further genetic studies are currently being performed. CPPD-CDD has been considered one of the great mimickers in rheumatology. It remains to be seen if this is a new disease or an unusual familial variant of DISH or CC.

Future Studies

Many families with monogenic segregation of CC have been reported. In most cases, no effort has been made to identify the gene involved, which is a pity, because this is a becoming relatively straightforward process and these families provide great insights into disease causation.

Great advances have been made in human genetics, making the identification of genes involved in common human diseases a realistic prospect. Notably, the development of high-throughput, high-density single nucleotide polymorphism genotyping platforms has enabled genome-wide association studies and the potential to screen all common human genetic variants for involvement in disease. These studies require large cohorts (> 1000) of well-characterized cases. As costs fall, their use in a wider range of diseases will become a possibility. Given the clear role of genetics in some forms of CC and the current absence of an effective treatment, a strong case can be made for performing such a study in this disease. Investigators must be encouraged to establish such a collection to enable a definitive study of apparently "sporadic" CC.

Conclusions

Although the causes of familial CPPD-CDD are still incompletely understood, there has been an enormous and fruitful interest in the etiopathogenesis of this disorder in recent years. The rapid development of genetic analysis is bringing new and interesting insights to this theme, providing strong in vivo evidence of the importance of bioregulation of PPI levels in controlling mineralization and identifying some critical genes in this system. Many other candidate genes are being investigated, and it is probable that in the near future, novel therapeutic targets will be found. It remains to be seen if an efficient treatment for CPPD-CDD can be found in future years.

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ANNEXE 3

A Novel *LEMD3* Mutation Common to Patients with Osteopoikilosis With and Without Melorheostosis

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Abstract Recent studies have reported loss of function mutations in the *LEMD3* gene, encoding an inner nuclear membrane protein that influences Smad signaling, as a cause of osteopoikilosis, Buschke-Ollendorff syndrome, and melorheostosis. We investigated *LEMD3* in a three-generation family with osteopoikilosis from the Azores, an affected father and daughter from Ireland with osteopoikilosis (the daughter also had melorheostosis), and two other individuals from the UK with isolated melorheostosis. We found a novel C to T substitution at position 2032 bp (cDNA) in exon 8 of *LEMD3*, resulting in a premature stop codon at amino acid position 678. This mutation cosegregates with the osteopoikilosis phenotype in both the Azorean family and the Irish family. It was not detected in any of the six unaffected family members or in 342 healthy Caucasian individuals. No *LEMD3* mutations were detected in the two patients with sporadic melorheostosis. The *LEMD3* mutation reported was clearly the cause of osteopoikilosis in the two families but its relationship to

melorheostosis in one of the family members is still unclear. Perhaps unsurprisingly in what is a segmental disease, we did not find *LEMD3* mutations in peripheral blood-derived DNA from the two other individuals with sporadic melorheostosis. The nature of the additional genetic and/or environmental influences required for the development of melorheostosis in those with osteopoikilosis requires further investigation.

Keywords Buschke-Ollendorff · Hyperostosis · Inner nuclear membrane protein · *MAN1*

Osteopoikilosis (OMIM 166700) is an autosomal dominant skeletal disorder characterized by the presence of multiple osteosclerotic lesions in different parts of the skeleton [1]. It occurs equally commonly in men and women, becoming evident at variable ages, although its appearance before the age of 3 years is uncommon. The most frequently involved sites are the epiphyses and metaphyses of long tubular bones, the wrist, foot/ankle, pelvis, and scapulae [2]. Most reported cases have been found incidentally on radiographs taken for other purposes. It can occur in isolation or in association with other skin and bone lesions, most notably in Buschke-Ollendorff syndrome (BOS), which is characterized by disseminated connective tissue nevi and osteopoikilosis [3, 4].

Melorheostosis (OMIM 155950) may occasionally coexist with osteopoikilosis in the same individual or within families [5–8]. It is characterized by a “flowing” hyperostosis of the cortex of human bones, sometimes described as a “dripping candle wax” appearance. It affects mainly the diaphyses of long bones, the hands and feet, pelvis, and only rarely the vertebrae or ribs. Classically it has a segmental distribution often limited to a single limb,

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in which several bones may be affected, but it may less commonly have a more extensive but unilateral distribution [9]. It may be present at birth but more commonly develops in late childhood.

Recently, loss of function mutations in the *LEMD3* gene (also named as *MAN1*), encoding an inner nuclear membrane protein [10], have been identified as the cause of BOS and osteopoikilosis [11]. In some of these families melorheostosis occurred as an additional finding in some individuals but to date no *LEMD3* mutations have been identified in individuals with sporadic melorheostosis. We aimed to identify mutations in *LEMD3* in multicase families with osteopoikilosis and also in patients with sporadic melorheostosis.

Materials and Methods

Patients and Controls

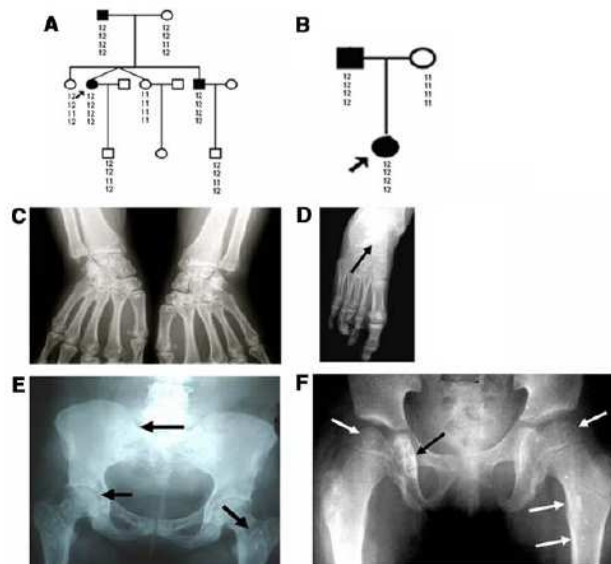
We studied a three-generation family (A) of Azorean origin with three members affected by osteopoikilosis (Fig. 1A). The proband, a 44-year-old woman who was diagnosed at the age of 23 years, was identified from patients attending the Rheumatic Diseases Clinic of Hospital de Santo Espírito, Angra do Heroísmo, Azores. Her non-identical twin sister had no radiographic evidence of osteopoikilosis. Two other affected individuals with osteopoikilosis were also identified in this family. We have also investigated four

patients attending the Nuffield Orthopaedic Centre, Oxford, UK. These included a small Irish pedigree (family B) in which the proband had melorheostosis, affecting predominantly the right leg and foot. She was diagnosed at the age of 12 years and had clear radiographic evidence of widespread osteopoikilosis, as did her father who did not have melorheostosis (Fig. 1B). Two individuals with isolated melorheostosis, affecting the left hand and the left foot, respectively, were also studied. All the individuals had a full skeletal radiographic survey (Fig. 1C–F). We also studied 342 healthy Caucasian subjects: 135 from the Azores and 207 from the UK. Genomic DNA was isolated from peripheral blood of patients, family members, and healthy controls after they had given their informed consent.

Mutation Screening and Genotyping

We screened all 13 *LEMD3* exons and flanking splice-sites using polymerase chain reaction (PCR) amplification, followed by direct sequencing of the PCR fragments (ABI Big Dye chemistry), using an ABI 3700 automatic sequencer (Applied Biosystems, UK). We first sequenced all the members of the Azorean family, the UK family, the two individuals with sporadic melorheostosis, and 24 healthy UK controls. Subsequently, we conducted limited direct sequencing of exon 8 in 207 UK and 135 Azorean controls to check that the mutation identified in patients was not a common polymorphism.

Fig. 1 A–F. Azorean (A) and Irish (B) pedigrees with osteopoikilosis showing (from top to bottom) the affected members and haplotypes for markers rs34604927, rs10534559, the novel mutation C/T, and rs3217456. The proband in the Irish family (B) also has melorheostosis. Radiographs of the proband in the Azorean family show “spotty” sclerotic lesions at the wrists (C), pelvis, and femur (black arrows) (E). Radiographs of the proband in the Irish family demonstrate melorheostosis at the right ankle (D), and there is bilateral evidence of osteopoikilosis in the proximal femora (white arrows) with unilateral melorheostosis affecting the right acetabulum (black arrow) (F)



Haplotype Analysis

We also carried out haplotype analysis for these two families by examining the sequencing data and phase-determination using GENEHUNTER-PLUS [12, 13].

Results

We identified a novel heterozygous mutation in exon 8 of *LEMD3* in both the Azorean and Irish families with osteopoikilosis. This mutation, a C to T transition at position 2032 bp (cDNA) (Fig. 2), changes the amino acid at position 678 from arginine to a stop codon; it co-segregates with osteopoikilosis in the Azorean family. In the Irish family, both the daughter and her father have the identical mutation to that found in the Azorean pedigree, but only the daughter has melorheostosis. None of the unaffected individuals in either family has this mutation. In contrast, no *LEMD3* mutations were detected in the coding region or exon-intron boundaries in the two patients with sporadic melorheostosis or any of the 342 controls.

Three other SNPs were also identified: the “t” deletion in intron 2 (rs34604927, 49 bp 5' of exon 3), “gatt” deletion in intron 7 (rs10534559, 30 bp 5' of exon 8), and “tttt” deletion in intron 11 (rs3217456, 25 bp 5' of exon 12). Sequencing data and haplotype analysis results are shown in Fig. 1A, B. The haplotype carrying the mutation is shown as 2222. The unaffected haplotypes are 2212 or 1111 and they are also the common haplotypes in the general population (http://www.ensembl.org/Homo_sapiens/geneview?db=vega;gene=OTTHUMG00000135148).

Discussion

In this study, we report a novel mutation in exon 8 of gene *LEMD3* causing a premature stop codon at amino acid position 678. Since none of the healthy controls or unaffected family members have this mutation it is clearly not a common polymorphism. Furthermore, the absence of any

other mutations in *LEMD3* in the affected members of these two families confirms that this mutation is the cause of the disease in these families. The observation that two further individuals with sporadic melorheostosis without a family background of osteopoikilosis do not exhibit *LEMD3* mutations is consistent with other reports [11, 14, 15]. This is perhaps unsurprising, given the segmental nature of the abnormality that might suggest a somatic mutation either in *LEMD3* or a gene with related functions.

It is of interest that we have found exactly the same previously unreported mutation in two geographically distant families. There is no known genealogical relationship between the two families but it seems likely that the observation can be explained by the nature of settlement of the Azores. During the fifteenth and sixteenth centuries these islands played an important role in commerce and slave trade between Africa, America, and Europe [16]. This strategic position between the three continents brought to the Azores people of many different origins, mainly Portuguese but also Jews, Moorish prisoners, black African slaves, Dutch (Flemish), French, Italians, English, and Spaniards. Recent studies corroborate the historical sources which confirm that the Azores were populated not only by the Portuguese but also by other Europeans, including the British [17–19]. Combining this historical information with our analysis of the disease associated haplotype in this study, we speculate that these two families share a common founder.

Loss-of-function mutations in *LEMD3* have previously been described in individuals with osteopoikilosis, irrespective of whether these patients had coexistent BOS or melorheostosis [11]. In contrast, no patients with melorheostosis alone were found to have *LEMD3* mutations. Similar results have been obtained in two previous studies [14, 15] as well as this study, indicating the absence of *LEMD3* mutations in cases of sporadic melorheostosis, using peripheral blood DNA sampling.

There have been sufficient reports of families and individuals with the co-occurrence of osteopoikilosis and melorheostosis to indicate that there is a likely causal link between the two disorders [5–8]. It is not known with certainty how frequently melorheostosis and osteopoikilosis occur together but from our experience it is not common. It seems likely that *LEMD3* is causally involved in melorheostosis but by mechanisms that require clarification. The segmental nature of the condition even when it occurs in the context of underlying osteopoikilosis is difficult to explain. Historically, melorheostosis has not been thought to be a heritable disorder although the segmental distribution hints at somatic mutation. In contrast, osteopoikilosis is a mild disseminated bone disorder clearly inherited as an autosomal dominant trait. By analogy with various autosomal dominant skin disorders for which a type

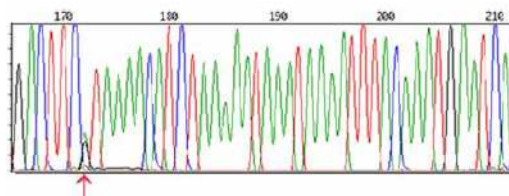


Fig. 2 The mutation (C to T, changing Arg to a stop codon) in exon 8 of *LEMD3* shown here as G to A by reverse primer direct sequencing

2 segmental manifestation has been postulated, Happle has suggested that melorheostosis may be best explained in such co-occurrence as a type 2 segmental manifestation of osteopoikilosis [20]. The *LEMD3* gene codes for a protein of the inner nuclear membrane that inhibits both the BMP and TGF β signaling pathways [11, 21]. Whether another genetic defect in this pathway is responsible for melorheostosis requires further study. The need to study tissue from the sites affected by melorheostosis itself has also been highlighted by our study and the preceding studies of others [11, 14, 15].

In conclusion, we have identified a new mutation in the *LEMD3* gene as the cause of osteopoikilosis and we have provided additional evidence that *LEMD3* mutations are not the sole cause of melorheostosis. We are currently trying to elucidate the underlying genetic defect(s) by studying a larger population of subjects with melorheostosis and obtaining affected tissues within which the disease mutations may be more relevant.

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ANNEXE 4

CHAPTER 3

Spondyloarthritis, Diffuse Idiopathic Skeletal Hyperostosis (DISH) and Chondrocalcinosis

Jácome Bruges Armas,* Ana Rita Couto and Bruno Filipe Bettencourt

Abstract

The authors describe the main clinical and radiological findings of common enthesopathic disorders—spondylarthritis (SpA), chondrocalcinosis/calcium pyrophosphate dehydrate crystal deposition disease (CPPD CDD) and diffuse idiopathic skeletal hyperostosis (DISH), stressing similarities and differences which may help in the differential diagnosis. They emphasize the clinical presentation of the “pseudoankylosing spondylitis” forms of CPPD CDD. They also review the most relevant genes and molecular mechanisms associated with these conditions and with another enthesopathic disorder with high prevalence in the Japanese population—ossification of the posterior longitudinal ligament (OPLL).

Introduction

Difficulties on the differential diagnosis between SpA and DISH may be relevant in the clinical practice, mainly because clinicians are frequently unaware of DISH manifestations. The classical acute Chondrocalcinosis manifestations—pseudogout, are usually a differential diagnosis of other crystal disorders like gout. However, other clinical and radiological manifestations of CPPD CDD, not very well known by the majority of the clinicians, namely those related to axial enthesopathic phenomena, may give origin to a wrong diagnosis. In this chapter we will update the genetics of these disorders, emphasising DISH and Chondrocalcinosis, because molecular knowledge of Ankylosing Spondylitis (AS), the major SpA subgroup, will be detailed in other chapters in this book. We will also evaluate the clinical and radiological findings in the three disease groups, indicating the similarities and differences between them and providing information which may allow an easier differential diagnosis.

Spondyloarthritis

Spondylarthritis, spondylarthropathy, or the spondylarthritides (SpA), are the most common terms to describe a group of diseases (Table 1),¹ which are characterised by the inflammatory involvement of the sacroiliac joints, peripheral inflammatory arthropathy and insertional tendinitis (enthesitis).^{2,3} The tendency towards familial aggregation is very well known⁴ and the association with HLA-B27 was described for the first time by Brewerton DA et al⁵ and Schlosstein LP et al⁶ Several criteria for Ankylosing Spondylitis was proposed, like the Modified New York criteria, 1984⁷ (Table 2). The criteria did not encompass conditions like Undifferentiated Spondylarthritis

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Table 1. Individual conditions that overlap to form the spondylarthritides

-
- Ankylosing spondylitis
 - Reiter's syndrome/reactive arthropathy
 - Enteropathic spondylitis
 - Psoriatic arthropathy
 - Uveitis
 - Juvenile ankylosing spondylitis
 - Undifferentiated spondylitis
 - Pustulotic arthro-osteitis
-

and criteria for the entire group of SpA were proposed. The European Spondyloarthropathy Study Group criteria (ESSG) have a sensitivity and a specificity of 87% (Table 3).⁸

The prevalence of idiopathic AS, the major SpA group, varies within different populations and the prevalence of HLA-B27. The diagnosis of idiopathic AS implies always the exclusion of other SpA. The sex related prevalence results always reported higher values in males, but figures of male female of 3:1 or 2:1 are acceptable. Hospital based studies concluded that the prevalence of AS was about 0.10-0.20%.^{9,10} The prevalence of AS based in studies of blood donors varies from 0.10-0.20%,¹¹ and population surveys showed the highest prevalence in the male Haidas inhabitants from northern Canada, of whom 4-6% have AS.¹² In European populations the surveys showed some variability—from 0.10% to 1.4%,¹³⁻¹⁵ related possibly to the methodology applied and to the HLA-B27 prevalence. It was also estimated that the prevalence of AS in the HLA-B27 population may be as high as 5%.^{16,17} According to the information obtained in hospital based and population surveys, there are milder frequently underdiagnosed forms of AS in the population characterised by less restriction of spinal disease and lower incidence of peripheral arthritis.^{18,19}

Sacroiliac joint involvement is the hallmark of AS, but the inflammation also involves frequently the spine, hips and shoulders (both considered axial joints) and, less frequently, peripheral joints. The age of onset is usually after 24 years, but rarely after 45 years old.²⁰ Extra-articular structures may also be affected like the eye (uveitis), the heart and, less commonly, the lung. Chronic low back pain and stiffness is the most common clinical finding in AS. Pain starts insidiously and patients usually complain that it is worse during rest and sleep and that there is improvement with movement and exercise. Pain may radiate into both thighs, simulating sciatica, but usually alternates first between one side and the other, later becoming persistent. The pain intensity is variable and frequently episodes of back (buttock) pain alternate with periods of mild pain and stiffness; this probably represents the aggression of the sacroiliac joint. Some less severe forms of disease may have milder or no back pain, stiffness and later, minor spinal restriction findings.^{19,21}

The involvement of shoulders and hips may occur in one third of patients. The hip is the second most frequent area affected by AS, the aggression occurs mainly in the first ten years after the

Table 2. Modified New York criteria, 1984

Criteria

1. Low back pain for at least three months' duration improved by exercise and not relieved by rest
 2. Limitation of lumbar spine motion in sagittal and frontal planes
 3. Chest expansion decreased relative to normal values for age and sex
 - 4a. Unilateral sacroiliitis grade 3-4
 - 4b. Bilateral sacroiliitis grade 2-4
- Definite ankylosing spondylitis if
(4a OR 4b) AND any clinical criterion (1-3)
-

Table 3. European spondyloarthropathy study group criteria

Inflammatory spinal pain or Synovitis (asymmetric, predominantly in lower limbs)
And any of the following:
• Positive family history
• Psoriasis
• Inflammatory bowel disease
• Alternate buttock pain
• Enthesopathy
Sensitivity, 77%; specificity, 89%
Adding:
• Sacroiliitis
Sensitivity, 87%; specificity, 87%

diagnosis and is usually bilateral.²² Shoulder girdle (glenohumeral, acromioclavicular or sternoclavicular joints) disease may lead to mild pain and restriction of movements to severe ankylosis of the joint.²³ Peripheral arthritis is usually asymmetric, oligoarticular, transient, mild, rarely persistent and usually without permanent deformities or erosions.²⁴ Enthesitis may be found in justa-articular or extra-articular insertion tendon or ligament areas and causes pain and tenderness. Costosternal junctions tenderness can cause chest pain and tendinitis of the Achilles tendon may cause severe and longlasting pain.²⁵ The role of proinflammatory cytokines in the development of osteoporosis was raised, after confirmation of low lumbar and femoral neck reduction in bone densitometry by dual energy X-ray absorptiometry (DEXA) in early AS.²⁶ Osteoporosis in patients with severe AS of long duration may possibly result also from the absence of spinal mobility and is a cause of fracture that occurs predominantly in the cervical spine.²⁷

The most common extra skeletal manifestation of AS is acute anterior uveitis (AAU) which occurs in about 30% of patients and is much more frequent in the presence of HLA-B27. The uveitis is usually unilateral, frequently recurrent and subsides if left untreated in 2-3 months, with no sequelae. Symptoms start abruptly—pain, photophobia and blurred vision are the main manifestations. Heart disease occurs mainly in patients with longstanding AS and peripheral arthritis.²⁸ Several manifestations have been described, from the complete set of cardiac conduction abnormalities to aortitis of the ascending aorta and aortic valve incompetence.

The severe forms of AS usually evolve within a period of 10 years and the characteristic thoracic kyphosis, posture and gait make easier the diagnosis. In these later stages the evident limitations of spine movement are mainly related with ankylosis, following inflammatory involvement of the interapophyseal spine joints and spine enthesopathic calcification. Earlier radiographic manifestations of the inflammatory process start usually in lumbar spine, affecting the superficial layers of the annulus fibrosus where they attach to the corners of vertebral bodies, followed by reactive sclerosis and erosive bone resorption, X-rays show squaring of the vertebral bodies, ossification of the superficial layers of the annulus fibrosus and bridging between vertebrae (syndesmophytes).²⁹ The apophyseal joints are involved frequently in AS and ankylosis is usual and the posterior ligaments may ossify causing also ankylosis of one spine process to another.³⁰ Enthesopathic calcification and ossifications are frequent in AS mainly at sites of osseous attachment of tendons and ligaments. Ischial tuberosities, the iliac crest, but also the calcanei and the femoral trochanters may have evidence of previous erosions and osteitis. Ankylosis of the peripheral joints is a rare event in AS. The sacroiliac joint is usually the first affected joint in AS. The earliest radiographic signs consist of bilateral blurring of the subchondral bone plate, followed by erosions and sclerosis in the iliac side of the joint. Later there is fibrosis, calcification, interosseous bridging and ankylosis. Some patients may have inflammatory back pain for several years without X-ray findings, mainly women³¹ and in these occasions sacroiliac CT scan³² or MRI³³ may be helpful.

Diffuse Idiopathic Skeletal Hyperostosis (DISH)

Despite being a very frequent disease, whose first detailed description is attributed to Forrester and Rotes Querol,³⁴ there are still many unknown data about DISH. Clinical data is frequently not identified by clinicians and this is probably because their clinical manifestations were always regarded as mild and without important consequences. The majority of criteria developed to diagnose DISH is related to radiographic findings. The three criteria to diagnose spinal DISH are: (1) The presence of flowing calcification and ossification along the anterolateral aspect of at least four contiguous vertebral bodies with or without associated localised pointed excrescences at the intervening vertebral bodies—intervertebral disc junctions. (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 margin sclerosis. (3) The absence of apophyseal joint bony ankylosis and sacroiliac joint erosion, sclerosis, or intra-articular osseous fusion.³⁵

Albeit being a disease that affects mainly the axial skeleton and in particular the thoracic spine, it is now recognised that this condition is not limited to the spine, but involves frequently peripheral joints and for this reason the previous designation of Forestier and Rotes-Querol disease was changed to DISH. The disease is characterised by ossification of the anterior longitudinal ligament and by the presence of flowing osteophytes involving predominantly the right side of the thoracic spine, with preservation of the intervertebral space. Abnormalities in the sacroiliac joints are not uncommon; the upper part of the sacroiliac joint is ligamentous, while the lower 2/3 is a synovial joint and asymmetric superior intraarticular fusion, osteophytes with and without bridging and vacuum phenomena were described by Durbach et al³⁶ There are also reports on the intra-articular ankylosis of the sacroiliac joints, but this finding is controversial.³⁷ Pelvic bony proliferation (whiskering) is frequently found at the sites of ligaments and tendons attachment to bone, like the iliac crest, ischial tuberosity and trochanters. The iliolumbar and sacrotuberous ligaments may be calcified and ossified.

The disease prevalence varies but data from autopsy showed that there was a frequency of 28% in a population with an average age of 65 years.³⁸ The prevalence is higher in males in the majority of reports.^{39,40} Although patients are sometimes asymptomatic, pain and stiffness began usually in the thoracolumbar spine, may be initially intermittent, but can progress to involve other spine segments. Sometimes symptoms are reported many years before a radiographic diagnosis. Dysphagia, probably related with prominent cervical osteophytes is a frequent symptom in some series of DISH patients.⁴¹ There are several reports of patients between 20 to 40 years old with radiographic aspects that do not fulfill the classic DISH criteria, but the findings were strongly suggestive.⁴² Not infrequently these findings are related to pain over the entheses in peripheral joints, associated with radiological abnormalities. Enthesopathic phenomena are more frequent at the elbows—medial epicondylitis and olecranon, patella, heel and tibial spines.^{43,44}

There are several reports on the positive association of DISH and Diabetes Mellitus⁴⁵ and some reports in the literature claim on the familial aggregation of DISH and OPLL.⁴⁶⁻⁵³ OPLL, a common cause of spinal canal stenosis in Japanese population, is characterised by ossification of the posterior longitudinal ligament and has a prevalence of 1.9-4.3%.⁵⁴ DISH and OPLL may occur together and Resnick et al reported that about 50% of cases of DISH are associated with OPLL.⁵⁵

Chondrocalcinosis/Crystal Deposition Disease (CPPD CDD)

The term *chondrocalcinosis* (CC) refers to the calcification of joint hyaline or fibrocartilage, a frequent radiologic feature of calcium pyrophosphate dehydrate crystals deposition (CPPD) and is attributed to Zitnan and Sitaj.⁵⁶ Other designations have been coined like *pyrophosphate arthropathy* which is widely used in the United Kingdom,⁵⁷ although presently the most accepted name to describe the clinical syndromes associated with CPPD is CPPD crystal deposition disease.⁵⁸ In this chapter we have adopted the designation of CPPD CDD. This condition is usually reported as a disease of older patients which prevalence increases with the increasing of age. Three main groups were described—Sporadic forms which are considered the largest group, hereditary CPPD CDD

and CPPD CDD associated with metabolic diseases: hemochromatosis, hyperparathyroidism, hypothyroidism, hypophosphatasia, Wilson's disease and other entities. CPPD crystal deposition disease is frequently accompanied by acute episodes of arthritis involving the larger joints, mainly the knees—the pseudogout syndrome and these episodes are caused by the precipitation of CPPD crystals in the joint fluid.^{59,60}

The definite diagnosis is performed after the demonstration of CPPD crystals in tissues or synovial fluid by compensated polarised light microscopy. There is a diagnostic classification based on this assumption and modified to include radiographic findings suggested by Resnick et al⁶¹ and Martel et al,⁶² (Table 4).⁵⁸ The finding of CPPD crystals is crucial, because this disease is quite heterogeneous and may resemble other conditions, like gout, rheumatoid arthritis, neuropathic disorders and Ankylosing Spondylitis.^{58,63} McCarthy describes various clinical presentations of joint disease associated with CPPD deposition and several groups are considered—Type A-Pseudogout, Type B-Pseudorheumatoid Arthritis, Type C and D-Pseudo-osteoarthritis, Type E-Lanthanic (asymptomatic) and Type F-Pseudoneuropathic joints. About 75% of CPPD CDD patients presents with the pseudogout (Type A) pattern (25%), or with the Pseudo-osteoarthritis (Types C and D, 50%).⁵⁸ In this last group acute attacks occur in approximately half of the cases and the main joints affected are the knees, wrists, metacarpophalangeal joints, hips, spine, shoulder, elbows and ankles. Some difficulty to perform a differential diagnosis with osteoarthritis (OA) is usual, but CPPD CDD affects joints that are not frequently involved in primary OA, like the metacarpophalangeal joints, wrists, elbows and shoulders. The pattern of joint involvement is usually symmetric and flexion contractures of the affected joints, enthesopathic symptoms mainly in the Achilles, triceps and shoulders tendons and entrapment syndromes of the upper limbs have been described.^{64,65}

Table 4. Diagnostic criteria and categories for CPPD crystal deposition disease

Criteria	
I.	Demonstration of CPPD crystals, obtained by biopsy, necropsy, or aspirated synovial fluid, by definitive means; e.g., characteristic “fingerprint” by X-ray diffraction powder pattern or by chemical analysis
II.	A) Identification of monoclinic or triclinic crystals showing a weakly positive, or a lack of, birefringence by compensated polarised light microscopy. B) Presence of typical calcifications on radiographs
III.	A) Acute arthritis, especially of knees or other larger joints, with or without concomitant hyperuricemia. B) Chronic arthritis, especially of knee, hip, wrist, carpus, elbow, shoulder and metacarpophalangeal joints, particularly if accompanied by acute exacerbations; the chronic arthritis shows the following features helpful in differentiating it from osteoarthritis. 1) Uncommon site for primary osteoarthritis; 2) Radiographic appearance; 3) Subchondral cyst formation; 4) Severe progressive degeneration, with subchondral bony collapse (microfractures) and fragmentation, with formation of intraarticular radiodense bodies. 5) Variable and inconstant osteophyte formation. 6) Tendon calcifications, especially of Achilles, triceps and obturator tendons. 7) Involvement of the axial skeleton and subchondral cysts of apophyseal and sacroiliac joints, multiple levels of disc calcification and vacuum phenomenon
Categories	
A.	Definite—criteria I or II (A) and II (B) must be fulfilled
B.	Probable—criteria IIA or IIB must be fulfilled
C.	Possible—criteria IIIA or IIIB should alert the clinician to the possibility of underlying CPPD deposition

The radiographic features of CPPD CDD may be divided in articular and periarticular calcifications and cartilage, synovium, capsula, tendons, ligaments, soft tissues, blood vessels and bursae may be affected. Demonstrable articular cartilage calcifications are more frequent in knees menisci, symphysis pubis, triangular cartilage of the wrists, annulus fibrosus of the intervertebral disc and acetabular labra (hips).⁶¹ Synovial calcification is also common in CPPD CDD and is more frequent in the wrist particularly about the radiocarpal and inferior radioulnar joints, knees and MCP joints.^{66,67} Capsular calcification is most frequently observed in the elbows, metatarsophalangeal and metacarpophalangeal joints, as fine or irregular linear calcifications and joint contractures mainly in the elbows are frequently associated.⁶¹

The first description of familial CPPD CDD is attributed to Zitnan and Sitaj in 1963,⁶⁸ but many other families have been reported since then, in different countries and continents.⁶⁹⁻⁷⁵ Two distinct clinical phenotypes were described: a group has a milder phenotype and the symptoms start after 60 years old, the disease is frequently oligoarticular and is indistinguishable from the sporadic form of CPPD CDD. The other phenotype is characterised by early onset of symptoms, usually between 20 and forty, but younger ages were reported.⁷⁶ Polyarthritides is a frequent finding, the prognosis is variable but the evolution is frequently characterised by the severity of the clinical and radiological picture.^{68,72,76-78}

A systematic familial investigation of all cases with a diagnosis of sporadic CPPD CDD has not been performed and because of this, an exact figure of the prevalence of both forms is not known. According to McCarty a diagnosis of sporadic CPPD CDD should be done only after exploring family data.⁷⁹ The frequency of the hereditary forms is probably higher than reported and is due mainly to the lack of appropriate investigations. Rodriguez-Valverde et al⁸⁰ found a prevalence of 17.1% of hereditary CPPD CDD forms in Spain, McCarthy observed a 25% prevalence of familial disease in his patients with pseudogout⁷⁹ and Dapica and Gomez-Reino found a 28% in a Spanish urban population.⁸¹

Although the clinical and radiographic features, do not allow an easy distinction between the sporadic and the hereditary cases of CPPD CDD, the age of the first symptoms may be helpful. Furthermore, several reports in the literature point to an association of the CPPD CDD hereditary forms with DISH and ankylosis.^{49,76,78,82-87} Harris J et al³⁹ also describes 6 patients with CPPD CDD in knees and hips, out of 34 DISH patients. Although in this report the patients ages ranged from 47 to 83 years old, the author does not refer to the age of the six CC patients. Other reports describe the association of Chondrocalcinosis and Ankylosing Spondylitis, or a clinical presentation of Chondrocalcinosis mimicking Ankylosing Spondylitis, referring this form as "pseudoankylosing spondylitis."^{88,89}

Molecular Genetics

Mineral deposition is an extremely balanced process normally restricted to bone formation. It is a tightly controlled process taking place in chondrocyte and osteoblast derived matrix vesicles (MVs). These vesicles contain enzymes, calcium and inorganic phosphate ions (Pi) and it is within these vesicles that the first hydroxyapatite (HA) and CPPD crystals are formed.⁹⁰⁻⁹³ It is known that inorganic pyrophosphate (PPi) antagonizes the formation of HA crystals hence the importance of having a tight balance between the levels of extracellular Pi and PPi for normal mineral deposition.^{94,95} It has also been long known that individuals suffering from acute inflammatory reactions caused by calcium pyrophosphate dehydrate deposition have high concentrations of inorganic pyrophosphate in synovial fluids.⁹⁶ Much work has been performed on this subject given that knowledge of PPi metabolism has been considered of great importance in ectopic calcifications studies.

To date, many genes involved in mineral deposition have been identified.⁹⁷ Mutations in several of these genes affect the rate of generation, transport and degradation of pyrophosphate (PPi) leading to an unbalanced level which will ultimately lead to ectopic deposition of crystals throughout the skeleton⁹⁵ (Fig. 1).⁹⁸

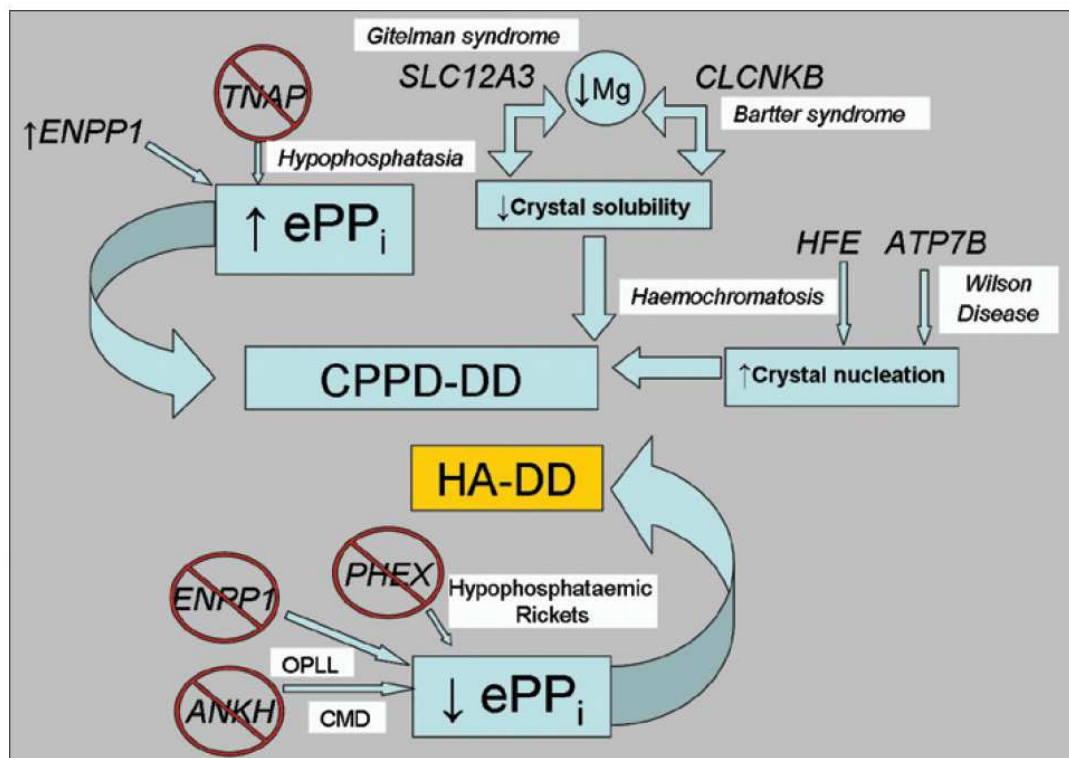


Figure 1. Mechanisms underlying associations of genetic diseases with ectopic mineral deposition. CMD-craniometaphyseal dysplasia; CPPD-CPP-calcium pyrophosphate deposition disease; ENPP1-enzyme ectonucleotide pyrophosphatase; ePP_i-extra cellular inorganic pyrophosphate; HA-CDD-hydroxyapatite crystal deposition disease; Mg-magnesium; OPLL-ossification of the posterior longitudinal ligament; TNAP-Tissue nonspecific alkaline phosphatase (adapted from: Couto AR, Brown MA. Genetic factors in the pathogenesis of CPPD crystal deposition disease. *Curr Rheumatol Rep* 2007; 9(3):231-6.

CPPD CDD segregates in an autosomal dominant manner and has been described in numerous families.^{49,99,100} Until now two chromosomal regions have been linked to CPPD CDD: chromosome 8q (CCAL1) and chromosome 5p (CCAL2).

In 1995, Baldwin and his colleagues,¹⁰¹ reported genetic linkage between markers on human chromosome 8q and the disease in a large family from Maine with early onset CPPD and severe degenerative OA [CCAL1, MIM 600668]. The disease causing gene was not mapped yet but there is a strong possibility that this gene is primarily related with osteoarthritis and that the CPPD deposition is secondary, enhanced by the degenerative changes in cartilage.

A second form of autosomal dominant CPPD CDD CCAL2 [CCAL2, MIM 118600] was initially described and genetically localised in five English families with CPPD CDD. Linkage was established to chromosome 5p, with a multipoint LOD score of 4.6.¹⁰² Subsequently, in two CPPD-CC unrelated families, one from Argentina and the other from France, another zone in chromosome 5p, mapping just centromeric to the first, was further identified through recombination analysis.¹⁰³ The identification of the *ank* gene and demonstration that loss-of-function mutations in the gene cause ectopic hydroxyapatite deposition in the ank mouse model,¹⁰⁴ led investigators to hypothesize that gain-of-function mutations in the human homologue of this gene may be responsible for the disease in these families.¹⁰⁵ This finding was confirmed in other families with autosomal dominant CPPD CDD.¹⁰⁶⁻¹⁰⁸

In 2005, Zhang et al¹⁰⁹ demonstrated that a common promoter region polymorphism is associated with "sporadic" cases of CPPD CDD. The polymorphism identified in this study, a -4 basepair G-to-A transition, was found in 4% of a 128 patient cohort with widespread premature

chondrocalcinosis and/or pseudogout.¹⁰⁹ So it was suggested that *ANKH* variants play a role in both familial and severe forms of 'sporadic' chondrocalcinosis.

The *ANKH* gene encodes a 492 amino acid multiple-pass transmembrane protein which is thought to be involved in the transport of inorganic pyrophosphate (PPi) across plasma membrane to the extra cellular compartment. Loss-of-function mutations in *ank* lead to an increase in intra cellular PPi and a decrease in extra cellular PPi, promoting the excess calcium hydroxyapatite formation found in the *ank/ank* mouse.¹⁰⁴

The exact mechanism by which *ANKH* mutations cause these diseases is unknown. The structure of the ANK protein has not been determined, possibly because of technical difficulties involved in structural studies of transmembrane proteins. Strong evidence has been presented that ANK is a PPi transmembrane transporter. The strongest evidence for this has been for hydroxyapatite-deposition associated mutations causing either the *ank* mouse phenotype, or craniometaphyseal dysplasia. Here, transfection studies and studies in transgenic mice have shown that these *ank* variants are associated with markedly reduced PPi transmembrane transport.¹¹⁰ With CPPD-associated mutations, the same group did not observe any significant difference in PPi transport compared with wild-type *ank*.¹¹⁰ Transient transfection studies in chondrocyte cell lines have suggested that not all CPPD CDD mutations lead to increased extracellular PPi and some have other effects on chondrocyte maturation and mineralization independent of effects on PPi.¹⁰⁹ The P5L mutation, associated with CPPD CDD, increases Type X collagen expression in both transient and stable transfection studies,^{109,111} suggesting an effect of the mutation on chondrocyte maturation, perhaps explaining the association of this mutation with chondrocalcinosis (CC).

Part of the difficulty in determining the function of ANK and effects of *ANKH* mutations may relate to the technically challenging nature of measurement of extra- and intra-cellular PPi and the fact that available chondrocyte cell lines are not very close matches to primary cells. Further, ATDC5 chondrocytic cell lines express high levels of alkaline phosphatase, which breaks down PPi and CH-8 chondrocytic cells express high levels of TNF, which has been shown to inhibit PPi elaboration by chondrocytes.¹¹² Primary chondrocytes de-differentiate rapidly in culture, complicating their use. The absence of a murine model of CPPD chondrocalcinosis is a particular weakness. Even hypophosphatasia mice, with severe chronic elevations of ePPi, do not develop CPPD chondrocalcinosis. This may be due to the relatively short life span of mice compared with humans, or some characteristic of murine cartilage. The development of such a mouse model would greatly advance our ability to investigate the aetiopathogenesis of this disease.

The association of *ANKH* with AS is also rather controversial. The *ank* mouse initially came to attention as a potential model of human AS. Further, reports of a clinical association between AS and another disorder of ectopic hydroxyapatite deposition, OPLL raised the possibility that *ANKH* may play a role in the pathogenesis of spinal ossification in AS. While a study performed by Tsui et al¹¹³ reported a modest association of ANKH-OR and ANKH-TR to AS susceptibility, a study performed by Timms et al¹¹⁴ showed no evidence of association of *ANKH* polymorphisms with either susceptibility to or severity of disease. Further genotyping 201 multiplex AS families with nine *ANKH* intragenetic and two flanking microsatellite markers and family-based association analysis, Tsui et al (2003)¹¹⁵ showed that the region associated with AS in women was only significantly disease associated in the test of interaction among the subset of families with affected individuals of both genders. These findings support the concept that *ANKH* plays a role in genetic susceptibility to AS and reveal a gender-genotype specificity in this interaction. To our knowledge, no other genes possibly associated simultaneously to AS and CPPD CDD were reported. HLA-B27 is a critical factor on AS susceptibility and 48 subtypes were identified until now. B*2705 has been found in most populations worldwide and is overrepresented in the Circumpolar and Subarctic regions of Eurasia and North America. The association of this allele as well as B*2702 and B*2704 with AS has been proven in case-control studies.^{116,117} AS cases were also reported in individuals with the alleles B*2701, *2703, *2706, *2708, *2710, *2714, *2715 and *2719, in different populations, but the true meaning of these associations remain unclear due to insufficient data.¹¹⁸⁻¹²² HLA Class I molecules bind and trigger cell-surface receptors

specified by Killer cell Immunoglobulin-like Receptors (KIR) genes. A recent study aimed at analysing whether KIR3DL1 and 3DS1 genes and their possible synergistic effect with HLA-B alleles, influenced the susceptibility to AS in B27 patients.¹²³ This investigation showed that the presence of KIR3DS1 or KIR3DL1 in combination with HLA-B*27s/HLA-Bw4-I80 genotypes may modulate the development of AS. The susceptibility to AS could be determined by the overall balance of activating and inhibitory composite KIR-HLA genotypes. As a result of whole genome linkage screens several chromosomal areas possibly involved in disease susceptibility, or related with the age of symptom onset or to BASFI (Bath Ankylosing Spondylitis Functional Index) were identified.¹²⁴⁻¹²⁶ IL-1 cluster includes the genes for the cytokines IL-1 alpha, IL-1 beta and their inhibitor IL-1 receptor antagonist. These genes lay on chromosome 2 (2q) and were identified in one genome wide screen as AS associated.¹²⁵ In 2004 Timms et al showed that IL-1B -511 SNP and IL-1F10.1 were consistently AS associated even controlling other associated markers.¹²⁷ More recently the results of a metaanalysis involving groups from ten countries were published. Strong association was observed with three SNPs in the IL1A gene (rs2856836, rs1756, rs1894399, $P = 0.0036, 0.000019, 0.0003$ respectively). Two new loci related to AS, ARTS1 and IL23R, were reported in 2007 by the Wellcome Trust Case Control Consortium. The authors have genotyped 14,436 nonsynonymous SNPs and 897 major histocompatibility complex (MHC) tag SNPs from 1,000 independent cases of AS. They also claim that IL23R may be a common susceptibility factor for the major "seronegative" diseases.¹²⁸

There are genetic evidences suggesting the involvement of the enzyme ectonucleotide pyrophosphatase 1 (ENPP1) in ectopic calcification disorders such as the OPLL of the spine¹²⁹ and arterial calcification.¹³⁰ The normal activity of this enzyme, a membrane bound glycoprotein which produces inorganic pyrophosphate from ATP, inhibits pyrophosphate related calcification and mineralization. The *ENPP1* gene maps to chromosome 6 (6q22-q23) and comprise 25 exons that encode a protein expressed in a variety of tissues including matrix vesicles of bone and cartilage. The mouse model of OPLL, "tiptoe walking" (*ttw*) mouse, results from a naturally occurring nonsense mutation in the gene encoding ENPP1 (*Enpp1*^{-/-}). It exhibits ossification of the spinal ligaments and articular cartilage calcification by hydroxyapatite deposition.¹³¹ Mutations in *ENPP1* cause the condition idiopathic infantile arterial calcification, a condition characterised by periarticular and arterial hydroxyapatite deposition, with low activity of ENPP1 and low PPi levels.¹³² It is thus possible that gain-of-function mutations in this gene are involved in the development of CPPD CDD, though preliminary studies do not identify a major effect on CPPD CDD risk from common genetic variants of *ENPP1* (see below).¹³³

TNAP, an isozyme of a family of four homologous human alkaline phosphatase genes, is present in the matrix vesicles and has the ability of hydrolyze PPi. In humans, this enzyme is encoded by the gene *TNAP*, that maps in chromosome 1, containing 12 exons. Deactivating mutations in the *TNAP* gene cause hypophosphatasia [MIM 241500 (Infantile form), 146300 (Adult type)], characterized by poorly mineralized cartilage and bones, spontaneous bone fractures, CPPD CDD and elevated concentrations of PPi.¹³⁴

Using double knockout mice, lacking both *TNAP* and *PC-1* (*ENPP1*) genes, Hesse et al have shown evidences that TNAP and ENPP1 are key regulators of bone mineralization suggesting that these genes are antagonistic regulators in this process.¹³⁵

To evaluate the hypothetical role of *ENPP1* and *TNAP* in CPPD-CDD, Zhanget al¹³³ screened variants of *ENPP1* and *TNAP* in 128 sporadic Caucasian CPPD CDD patients and 600 healthy controls. No significant difference was observed in the alleles or genotype frequencies between patients and controls suggesting that these genes are not major genetic determinants of sporadic CPPD CDD susceptibility in white Caucasians.¹³³

A recent investigation performed by Bruges Armas et al in the Azores, Portugal, identified a series of families characterised by both CPPD CDD and florid ligamentous calcification, consistent with the condition DISH [MIM 106400].⁴⁹ The identification of CPPD crystals in all synovial fluids examined suggests that these families have a form of autosomal dominant CPPD deposition disease; however unlike previous reports of this condition, in these families, the CC was in all

cases complicated by peripheral and axial enthesopathic calcifications. Linkage to chromosomes 5p15 (CCAL2) and chromosome 8p (CCAL1) was excluded by microsatellite marker studies. Further genetic studies are currently being performed. It is well known that CPPD CDD has been considered as one of the great mimickers in Rheumatology. It remains to be seen if whether this is a new disease or, on the contrary, an unusual familial variant of DISH or CC.

The cause of DISH remains unknown; however, its frequent association with several metabolic conditions may give some clues concerning its aetiopathogenesis. Some biochemical facts have been observed in DISH patients which includes a higher insulin and growth hormone levels, possibly explaining the osteoblast cell growth and proliferation,¹³⁶ a greater body mass index (BMI), a higher serum level of uric acid and a higher possibility of having diabetes mellitus.¹³⁷ The association of DISH with diabetes mellitus has been much investigated in recent years.¹³⁸ It is believed that the consequence of the previously mentioned biochemical facts in the etiopathogenesis of DISH is the abnormal cell growth/activity in the bony–ligamentous region.^{45,139} It is known that insulin like growth factor (IGF)-I stimulates alkaline phosphatase activity and Type II collagen in osteoblasts and that growth hormone can induce the local production of IGF-I and IGF binding proteins in chondrocytes and osteoblasts.

Matrix Gla Protein (MGP) is an 84 residue vitamin K dependent protein expressed in various tissues and upregulated in bone cells. The MGP gene spans 3.9 kb and consists of 4 exons separated by 3 large intervening sequences which account for more than 80% of the gene.¹⁴⁰ It is believed that MGP action includes the inhibition of mineralization by suppression of bone morphogenetic protein 2 (BMP-2) which is a potent osteogenic factor. Sarzi-Puttini et al reported higher serum MGP concentration levels in male and female DISH patients, when compared with healthy controls. This group suggests that MGP may be a marker of hyperostosis because it is produced in larger amounts by patients with hyperostosis—inducing osteometabolic syndromes such as DISH.¹⁴¹

DISH has also been frequently associated with another paravertebral ligamentous ossification; the ossification of the posterior ligament of the vertebral bodies (OPLL) to the point that some studies state that OPLL can be seen in up to 50% of the patients with DISH.^{55,142,143} 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.¹⁴⁴

There have been only a few genetic studies on DISH patients most of them based on its association with OPLL. There are contradictory results concerning the involvement of Human Leukocyte Antigen (HLA) alleles.¹⁴⁵ Koga and colleagues performed a genetic linkage study in 91 OPLL affected sib pairs and report a predisposing locus for OPLL, on chromosome 6, close to the HLA complex. After analysis of genetic variants it was suggested that the candidate gene COL11A2 may be associated with the disease.¹⁴⁶ Maeda et al continued to investigate the association of COL11A2 with OPLL and report an association of a gender-specific COL11A2 haplotype with the disease. However, this association could only be seen in male patients.¹⁴⁷

To elucidate the genetics basis of OPLL Okawa et al investigated the *ttw* mouse, a naturally occurring mutant which exhibits similar anomalies in the spinal ligaments to the OPLL patients.¹⁴⁸ Using a positional candidate-gene approach this group identified a nonsense mutation (Glycine 568 to a Stop Codon) in the *Npps* gene as the causation of *ttw* phenotype. The homologous human gene, currently known as ENPP1, encodes an enzyme that is responsible for the production of inorganic pyrophosphate, a major inhibitor of calcification, thus regulating soft tissue calcification and bone mineralization.¹³¹ In 2001, novel mouse genes associated with ectopic calcification were isolated by differential display method using the same mouse model—*ttw*.¹⁴⁹

The association of TGB1 with OPLL has been postulated due to its role in the growth and differentiation of cartilage, endochondral and membrane bone and skin. Some studies have shown significant associations between variants of the gene and the prevalence of OPLL in the cervical spine.¹⁵⁰ A later study examined the relation between the previously associated polymorphism (T869 > G) and the radiologic characteristics of OPLL. This study concluded that this polymorphism

is not a factor associated with the occurrence of OPLL but rather a factor related to the area of the ossified lesion.¹⁵¹

To identify new genetic loci related to OPLL Tanaka and colleagues performed a genomewide linkage study with 142 affected sib-pairs. After multipoint linkage analysis, evidence of linkage to OPLL was detected in 6 zones of different chromosomes. The best evidence of linkage was found on a zone of chromosome 21 that was extensively investigated, with single nucleotide polymorphisms (SNPs), for linkage and association studies. After case control comparison COL6A1 was the gene most significantly associated with OPLL.¹⁵²

Recently a large-scale study investigating the genetics of OPLL was performed with the objective of identify new susceptibility genes for OPLL and to re-examine previously reported associations. Thirty five candidate genes were genotyped in sporadic OPLL patients (n = 711) and controls (n = 896). Significant associations were found in three genes; the most significant association was found in *TGFB3* gene (P = 0,0004). Previously reported associations of *COL11A2*, *NPPS (ENPP1)* and *TGFB1* were not reproduced in this study.¹⁵³

Conclusions

AS, CPPD CDD, DISH and OPLL are four different entities characterised by ectopic calcification and ossification of soft tissues, in particular entheses and ligaments. Very little is known about the genetic determinants involved in the causation of DISH and CPPD CDD but, due to its high prevalence, the better understanding of these processes will surely improve human health care. This review highlighted what has been learnt from recent genetic studies about the processes involved in these different but somehow related ectopic calcification disorders. Although some clues may be evident in each particular disease, particularly with AS and CPPD CDD, genetic polymorphisms which could explain the clinical enthesopathic manifestations identified in all of them were not yet identified. With the possible exception of the *ANKH* gene which is CPPD CDD associated and also possibly associated with AS, no other genetic associations common to at least two of these diseases were identified.

Previous reports have highlighted the resemblance of spinal CPPD CDD, DISH and AS^{89,154} and it can be difficult in some cases to differentiate these conditions. There are several reports in the literature focusing in the differential and sometimes conflicting diagnosis between AS and DISH and between AS and CPPD CDD. As already stated above, enthesopathic phenomenon are the main manifestations of these diseases and particularly in the axial skeleton they may be very similar, even in pathological specimens. Furthermore, the radiographic abnormalities that were identified by several authors in the axial component of CPPD CDD, with the exception of disk calcification, have not been enough stressed. For this reason, these radiographic manifestations are frequently confused with other conditions and usually are not considered in the differential diagnosis of axial enthesopathy. Several investigations, already cited in this chapter, report CPPD families with particular phenotypes which can be confounded with AS or with DISH. Of particular relevance are the reports of Reginato in the Chiloe Islands from Chile, van der Horst in Holland and Bruges Armas in the Azores, Portugal. It is interesting that patients from Argentina are the descendents of the islanders that in the 16th century admixed with Portuguese and Spanish, which came from Iberian Peninsula. The Azores (Terceira Island) where CPPD CDD/enthesopathic calcifications seems to be an endemic manifestation, in a familial context, were a secure port for ships demanding or coming from South America and were populated with people from different parts of Europe, namely an important group of Flemish whose connections with the Dutch are well known in the European history. It is possible that the descriptions in these three papers may be associated with migrations of people across continents. These “pseudoespondylitis” forms may show an axial phenotypic expression very similar to AS (Fig. 2) and are frequently diagnosed in a subset of early-onset familial CPPD CDD. These patients are symptomatic, have frequently peripheral arthritis and frequent pseudogout attacks, sometimes with complete articular destruction and ankylosis (Fig. 3).

Axial disease of CPPD CDD simulating AS or DISH was very well documented in a series of pathology reports by Resnick et al.¹⁵⁵ Osteolysis and subluxation of the cervical spine, changes in the apophyseal joints, “vacuum” phenomena and collapse of multiple disks and calcification of the odontoid process, interspinous bursae, or ligamentum flavum are some of the findings which show the resemblance with AS. According to these authors, they documented the widespread identification of CPPD crystals in the spine and sacroiliac joints and there appears to be a definite relationship between crystal deposition and adjacent structural joint damage in axial and peripheral joints and entheses. The initial site of crystal deposition are the outer fibers of the annulus fibrosus,

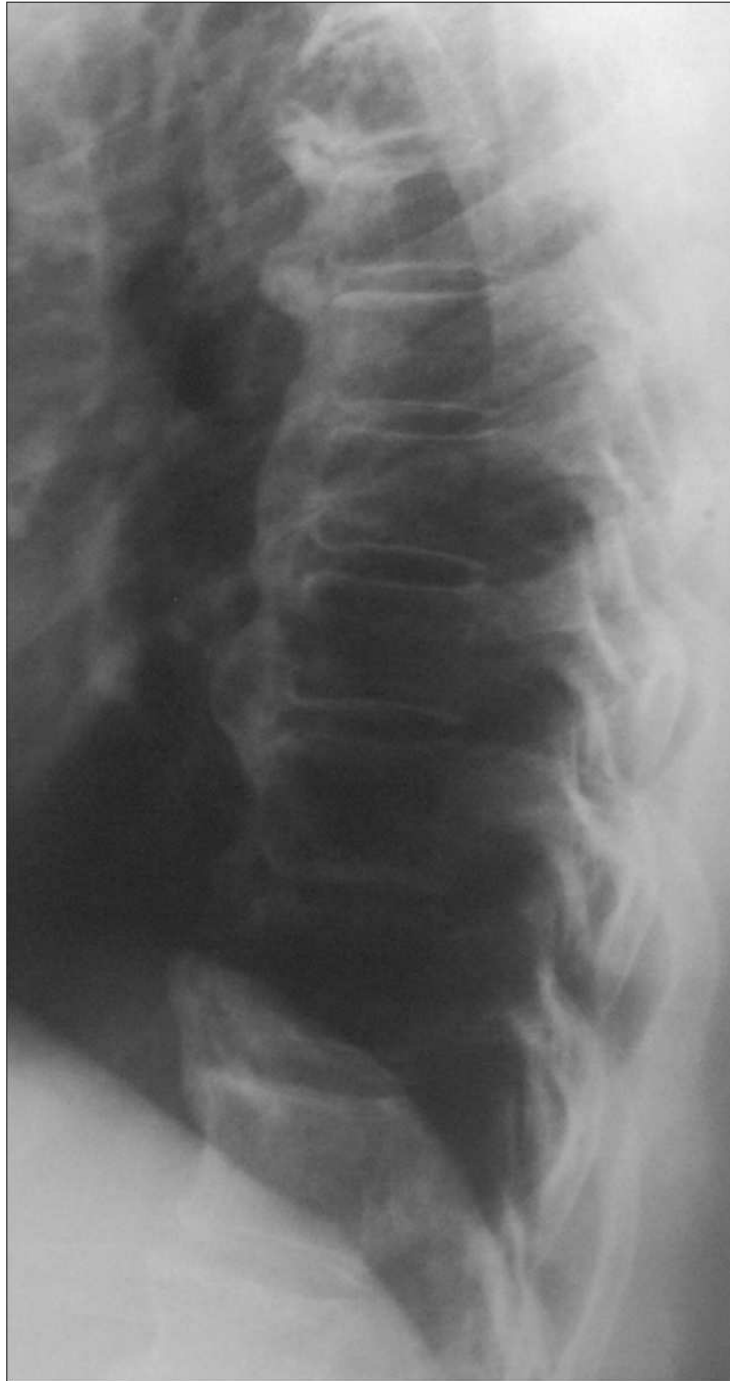


Figure 2. Dorsal spine calcification simulating AS in a patient with CPPD CDD.

producing syndesmophytes very similar to AS and further crystal deposition may be responsible for the other phenomenon like extensive disc calcification. The synovium lined apophyseal joints which are very frequently involved by the inflammatory process in AS, are also injured by the pro-inflammatory consequences of the deposition of CPPD crystals in these joints. Calcification of the cartilage, synovium and capsule, joint space narrowing, bone sclerosis and ankylosis were all described in these joints. The sacroiliac joint invariably involved in AS, allowing the differential diagnosis with other conditions, may have subchondral erosions and reactive sclerosis in CPPD



Figure 3. Ankle ankylosis. The patient had axial “pseudoankylosing spondylitis” and peripheral ankles, knees and wrists arthritis.

CDD, simulating AS.^{62,155,156} Furthermore, inflammatory back pain has been described in some of these families, sometimes earlier in the disease, when a radiographic diagnosis is not possible, making the differential diagnosis more difficult.⁴⁹ In these cases, peripheral articular manifestations are useful to make a differential diagnosis, because peripheral destruction, bone fragmentation and ankylosis are rare in AS.

It is interesting to stress that the majority of the authors that reported CPPD CDD familial cases, believe that the disease is autosomal dominant. Because of this and also because some of the more severe CPPD cases reported in the literature were born in small villages or communities, where inbreeding was common, it may be hypothesized that they represent the homozygous manifestations of this disease.

The differential diagnosis between DISH and AS has been the subject of discussion in multiple publications. A majority of authors emphasizes the similarities of the spinal radiographic features between both diseases. Indeed, when a patient is diagnosed after 60 years old, the differential diagnosis may be difficult. In these ages, when fusion already occurred in AS, back pain may not be a relevant symptom and spine X-rays may show a similar picture in both diseases. DISH criteria were set to allow the differential diagnosis. The absence of ankylosis, erosion, sclerosis, or intra-articular osseous fusion of apophyseal joint, is an important criteria to diagnose DISH. Also, another criteria which should allow the differential diagnosis between DISH and AS is the sacroiliac disease, always present in AS patients on their middle ages. However it is important to stress that sacroiliac abnormalities may be found in DISH, although erosions and intra-articular bony ankylosis are rarely present. Pelvic X-rays may be suspicious of sacroiliac disease and CT scans may show partial superior bilateral or unilateral bridging osteophytes or fusion.³⁶ Other authors reported progressive sacroiliac obliteration and ossification of the sacroiliac joints in DISH, but this findings are not usual.^{37,157} The peripheral musculoskeletal manifestations of DISH are usually not stressed in the literature. DISH is a diffuse disease and several enthesal extraspinal sites may be affected. They may also help in the differential diagnosis, because there is usually a close clinical-radiographic correlation. New bone formation is frequently found in places where there is pain and sometimes swelling. These findings have a high prevalence in heels and elbows where enthesophytes may be identified on X-rays.⁴⁴

Although it is clear that recent research revealed different molecular mechanisms underlying the pathogenesis of these conditions, the molecular basis of the enthesopathic/ossification phenomena which is a common finding of AS, CPPD CDD and DISH, is not know. Future research will address this issue and may possibly clarify the reason why some radiographic features of these diseases are quite similar.

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ANNEXE 5

The coexistence of ankylosing spondylitis and diffuse idiopathic skeletal hyperostosis—a postmortem diagnosis

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Abstract A 72-year-old male was diagnosed as having ankylosing spondylitis, mainly according to radiological findings, confirmed as HLA-B27-negative. Postmortem examination of the skeleton raised doubts on the initial diagnosis, since spinal findings pointed out also to diffuse idiopathic skeletal hyperostosis. The authors discuss the differential diagnosis and enhance the postmortem findings which allowed the diagnosis of the two clinical entities in the same patient.

Keywords Bone pathology · Joint fusion · Sacroiliitis · Spondyloarthropathy · Syndesmophytes

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Introduction

Ankylosing spondylitis (AS) and diffuse idiopathic skeletal hyperostosis (DISH) are both complex disorders of unknown etiology involving mainly the axial skeleton with bony proliferation and ankylosis, although both can also display enthesopathic manifestations in the peripheral skeleton. Despite these similarities, AS is a rare chronic inflammatory disorder (0.23–1.8% in Europe) of earlier age onset and with inflammatory manifestations, whereas DISH is usually described as a more common skeletal condition (6–12% of routine autopsies) of elderly patients, with minor clinical manifestations [1–6]. Calcium pyrophosphate dehydrate crystal deposition disease (CPPD CDD) is another condition which involves the axial spine, being frequently undiagnosed. The most know finding is disc calcification, but syndesmophytes and apophyseal joint space narrowing, sclerosis and ankylosis are also frequent findings [7, 8].

The aim of this paper was to report a patient with sacroiliac and spinal disease diagnosed as AS late in life and confirmed as HLA-B27-negative. X-ray findings in the axial skeleton could also allow a DISH diagnosis. The authors had the rare opportunity to examine the skeletal remains years after the patients death. The differential diagnosis is discussed, taking into account the new information obtained.

Case report

Sacroiliac and spinal disease was identified in a 72-year-old male from Terceira Island (Azores, Portugal) during the European Vertebral Osteoporosis Study radiologic cohort [9]. He and his family members were further interviewed and examined for evidence of arthritis at the Rheumatic

Diseases Clinic of the Hospital de Santo Espírito (Angra do Heroísmo, Terceira Island). This patient had been a farmer since a young age, and his main complaints were related with ischaemic heart disease, which was the cause of death

when he was 78 years old. He had dorsal and lumbar back pain, at least since he was 40 years old, but he was unable to properly characterise his back pain. He had no peripheral joint disease, uveitis, psoriasis or inflammatory bowel



Fig. 1 Macroscopic images: **a** Antero-posterior view of the pelvic bones with the para-articular bony bridging on the sacroiliac joint. **b** Right lateral view of thoracic spine. Notice the flowing and undulating calcification along the anterior aspect, the calcification of supraspinous ligaments and the fusion of some costovertebral joints. Radiology images: **c** anteroposterior X-ray of the pelvic bones showing symmetric

sacroiliac fusion. **d** Lateral X-ray of the thoraco-lumbar column showing the integrity of the inter-vertebral disc height and discovertebral junctions. **e** Erosion and ankylosis of the whole sacroiliac joint on CT. **f** T10 intra-articular ankylosis of apophyseal and costovertebral joints (CT)

disease. Anteroposterior spine and pelvis X-rays suggested a diagnosis of ankylosing spondylitis, mainly because of bilateral fusion of the anterior and posterior components of the sacroiliac joint. His brother, an 80-year-old male, had dorsal spine DISH on X-rays, but no sacroiliac disease. The patient had four offspring, three males without symptoms and a 46-year-old daughter who had bilateral sacroiliac sclerosis and bilateral enthesopathic tricipital calcification. She had episodes of dorsal inflammatory-like pain, although she also complained of dorsal non-inflammatory pain. Routine blood tests of the patient and family members were normal, and HLA-B27 typing resulted negative.

Several years after the patient's death, the skeletal remains were morphologically examined by paleopathology experts, and postmortem X-rays and computed tomography (CT) scans were taken. Postmortem examination was made using the complete skeleton. No particular findings were observed in the appendicular skeleton, namely periostitis, erosions or enthesopathic phenomena, but several findings were observed in the axial skeleton (Fig. 1).

On pelvis examination, bilateral sacroiliac joint fusion and para-articular bony bridging were confirmed. Furthermore, X-rays showed a severe bilateral and symmetric sacroiliitis, and the CT study showed intra-articular ankylosis and erosion of the whole sacroiliac joint.

The anterior aspect of the spine displayed a flowing and undulating calcification along the anterior and right lateral aspect of T6–L5 vertebral segments. Furthermore, bony bridging between C4 and C5 was observed. The intervertebral disc height was normal in all segments, and there was no involvement of the discovertebral junctions.

Degenerative disease and ankylosis of some apophyseal and costovertebral joints were observed in the posterior aspect of the spine. Specifically, CT images showed intra-articular apophyseal joint fusion between C4 and C5, T7 and T8 and T9 and T11. Intra-articular costovertebral joint fusion in T10 and T11 was identified on the right side. Additionally, there was calcification of interspinous and supraspinous ligaments between T6 and T11.

In addition to the morphological examination, several bone samples including the calcified anterior longitudinal ligament were examined in a JEOL JSM-6301F scanning electron microscopy (SEM) that was coupled to a Noran Voyager X-ray elemental microanalyser (XRM) with energy dispersive spectrometry detection system, thus allowing in situ elemental analysis.

The SEM imaging was used to detect crystal structures in the bone samples. Several calcium crystal shapes are reported in the literature [7], including pin-like, rod-like, granular or rectangular, but none of these was observed in the bone samples. An elemental analysis of different regions of the ligament was also performed. Figure 2 shows one of these analyses, for which the relationship

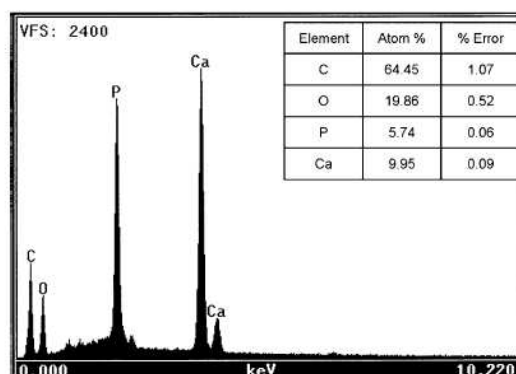


Fig. 2 X-ray elemental microanalysis spectrum of the calcified anterior longitudinal ligament. The calcium/phosphorus ratio was 1.73 ± 0.04 (expected value for calcium pyrophosphate—1.0)

calcium/phosphorus was 1.73 ± 0.04 , confirming the absence of calcium pyrophosphate crystals (expected value for calcium pyrophosphate—1.0). All the other spectra confirm a ratio of 1.70 ± 0.08 .

Discussion

Although bilateral sacroiliitis is a strong radiological evidence of AS, sacroiliac joint fusion may occur in both AS and DISH even if ankylosis of the synovial compartment of the sacroiliac joints is rarely observed in DISH [1, 2, 10–12]. DISH para-articular osteophytes may simulate intra-articular bone fusion on X-rays of DISH patients, and they are easily identified with sacroiliac CT scan [13].

The calcification or ossification encompassing the annulus and the anterior longitudinal ligament, which involves mainly the right side of the dorsal spine, are the classic features of DISH. The preservation of intervertebral disc space height, which is another classic finding in DISH, is sometimes unhelpful in the differential diagnosis mainly in old patients with concomitant osteoarthritis. In AS, calcifications or ossifications within the peripheral portion of the annulus fibrosus and erosive vertebral abnormalities are the main findings, although in longstanding cases, the anterior longitudinal ligament may become involved. Furthermore, the involvement of the apophyseal and costovertebral joints and the interspinous and supraspinous ligaments are frequent findings in AS [1, 2, 5, 6, 10, 13].

The antemortem data available in this case were not enough to help us to unambiguously establish the diagnosis. The patient was observed for the first time when he was 72 years old and was not able to remember his past symptoms. X-rays and CT scans were observed by several experts and a diagnosis of idiopathic AS was made,

although he was not HLA-B27-positive. The association of this allele with AS is at least 95% [3, 14]; although this association is not as strong as with other spondyloarthritis, the patient had no psoriasis nor inflammatory bowel disease.

According to ante and postmortem data, a diagnosis of coexistence of AS and DISH was proposed in our case. The absence of peripheral arthritis and of CPPD crystals did not support the diagnosis of CPPD CDD. Sacroiliac, apophyseal and costovertebral ankylosis, and calcification of the interspinous and supraspinous ligaments were favourable to the diagnosis of AS. The absence of involvement of the discovertebral junctions and the flowing calcification of the anterior longitudinal ligament in the dorsal and lumbar spine are very frequent in DISH. This association is quite rare mainly because AS is not a common disease, and very few cases have been reported in the literature until now [6, 15]. Other family members had a DISH diagnosis, and although there are few reports of familial DISH, a recent report [5] shows that in Terceira Island, families with multiple members affected with DISH and/or chondrocalcinosis are common.

Sacroiliac CT scan allowed the confirmation of intra-articular bony fusion, and the findings observed in the postmortem examination (confirmed by CT scans) were crucial to diagnose this association. The coexistence of DISH and AS may be possible mainly if the patient is diagnosed in a geographic area where DISH has a high prevalence.

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Conflict of interest X Jordana, I Galtés, AR Couto, L Gales, M Damas, M Lima, J Bruges-Armas have no disclosures or conflicts of interest.

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