INTEC

International Network on Ectopic Calcification

Ectopic Calcification in disease and (healthy) aging

INTEC Kick-off meeting

October 3d 2022

Muziekcentrum De Bijloke, Ghent, Belgium

WELCOME !

Dear participant of this INTEC kick-off symposium,
ear colleagues, dear friends,

It is our pleasure to welcome you in one of the many beautiful historical sites of Ghent - the Bijloke - for this first symposium of the International Network on Ectopic Calcification (INTEC).

Here, on this domain, healthcare has always been the focus of attention, from the early middle-ages up to the first half of the previous century.

It seemed thus the most appropriate place not only to look back but also to look at the future of healthcare. And in that future, ectopic calcification will have a prominent place.

Very few diseases have such a profound impact on the natural history and prognosis of hereditary disorders, acquired diseases and aging as ectopic calcification. Because of this, it deserved to be recognized as a separate disease in itself, to be studied to understand and influence its underlying mechanisms and to become more broadly known among healthcare professionals, patients and the general public. INTEC highlights all of the above as the goals we hope to achieve in the years to come.

This meeting would not be possible without the financial support of Ghent University, all INTEC partners, our sponsors and the coordination team of INTEC.

On behalf of all of them, we wish you an exciting symposium and a great stay in Ghent!

Olivier Vanakker.
INTEC coordinator.

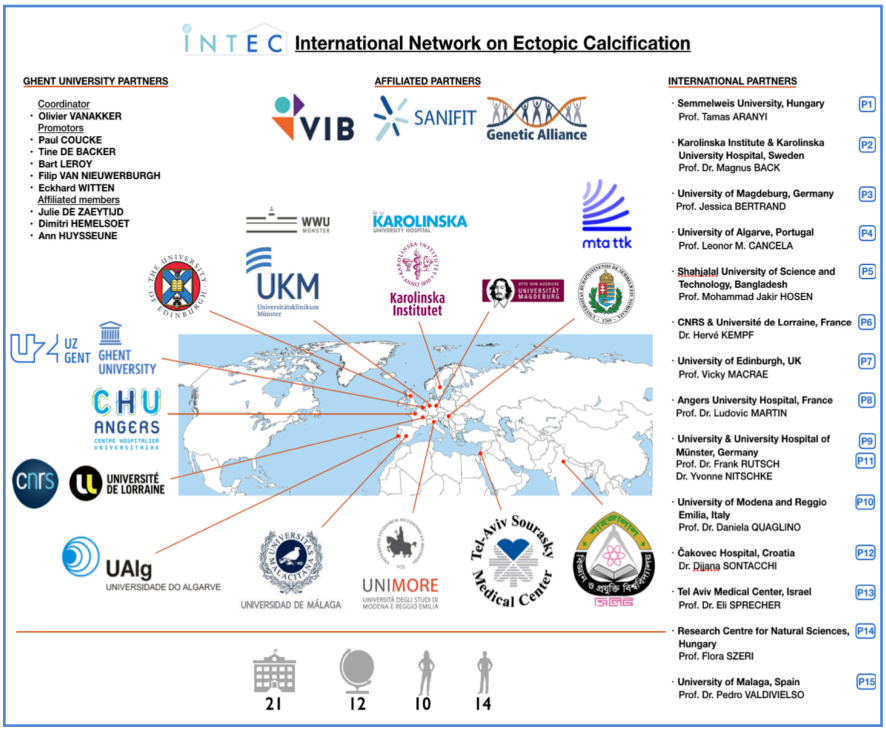
Karolien Aelbrecht
INTEC Project Manager.

ABOUT INTEC

The International Network on Ectopic Calcification (INTEC) is the latest of 13 **International Thematic Networks** which are being supported by Ghent University. These are cooperative networks consisting of Ghent University staff members and international partners concerning a specific topic of excellence in education and research.

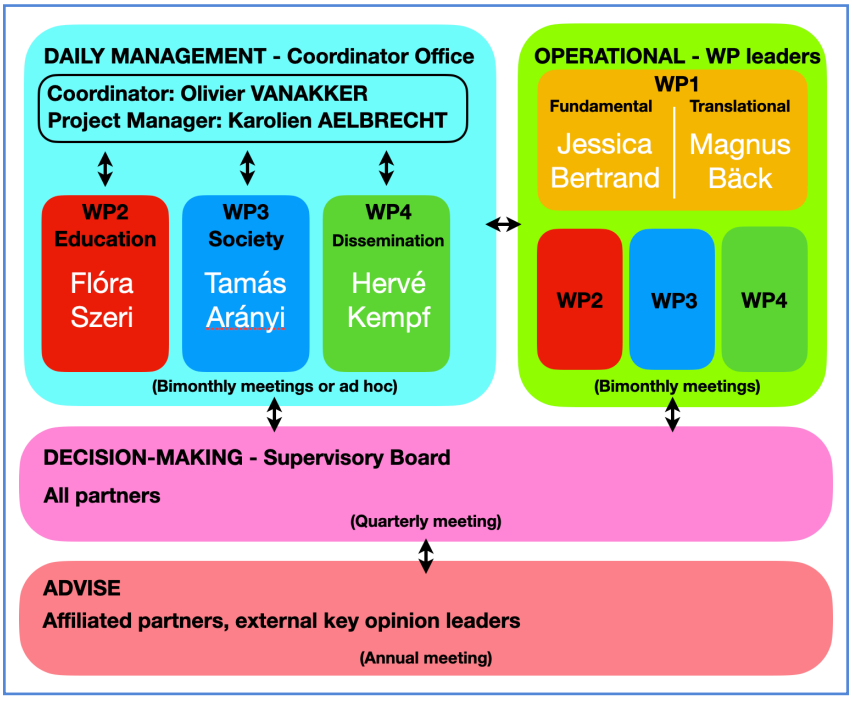
INTEC creates a robust network to **contribute to** the **advancement** of scientific knowledge and **unite** international institutions' expertise around ectopic calcification (EC). Ghent University (Hospital) has a strong history concerning connective tissue and EC research, being international leaders for more than three decades. A unique strength of the Ghent partners is the specific capacity for collaboration and integration over different disciplines and teams within the University (Hospital), such as clinics vs. molecular or basic research lab, ...

INTEC uses an interdisciplinary thematic approach to connect the relevant knowledge at Ghent University - fundamental and translational research, clinical and pharmaceutical experts with an established high-impact track record in studying aspects of EC - with an international network of **15 partners** who were carefully chosen to represent a diverse base of expertise, to exploit complementarities and to avoid competition. They are internationally recognized leading researchers that acknowledge the need for a collaborative approach to make important progress in the field of EC. Complementarity and diversity are achieved with respect to research expertise and education. In addition, important **stakeholders** are involved as affiliated members: Genetic Alliance - a coalition of patient organizations, an industrial partner - Sanifit, and VIB.



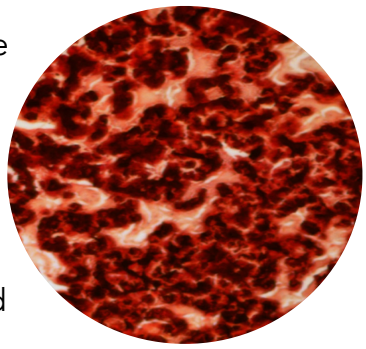
INTEC contains thus all necessary expertise to address the challenges of EC via **excellent and innovative science**, to invest in **high-quality education** of all EC stakeholders, and to establish sufficient **impact** for the network by dissemination of results. While the fundamental research partners have know-how and technological expertise necessary to accomplish INTEC's research aims, the clinical partners' expertise covers most tissues where EC occurs. In addition, it allows INTEC to take advantage of the largest cohorts of hereditary EC patients and integration of cohorts with acquired EC. These patients play a crucial role in the translation of basic science results.

INTEC is the largest consortium of experts dedicated to advancing the knowledge on acquired and genetic calcification towards clinical and therapeutic applications by stimulating, facilitating and enhancing cooperation and better transfer of knowledge as it bridges different disciplines (rare *versus* common disease, genotype *versus* phenotype, preclinical *versus* clinical) and sectors (academic research, clinical research and industrial R&D). We are confident that the network will fertilize a multitude of new collaborations and will broaden the perspective of all partners both in terms of internationalization and across sectors. In this respect, it is important that INTEC is an **open network** that welcomes all stakeholders relevant for EC in aging and disease. INTEC's collaborative ecosystem will reinforce the efficiency of the Research and Innovation on EC by decreasing fragmentation, avoiding duplication, and identifying major research gaps that can be tackled together.



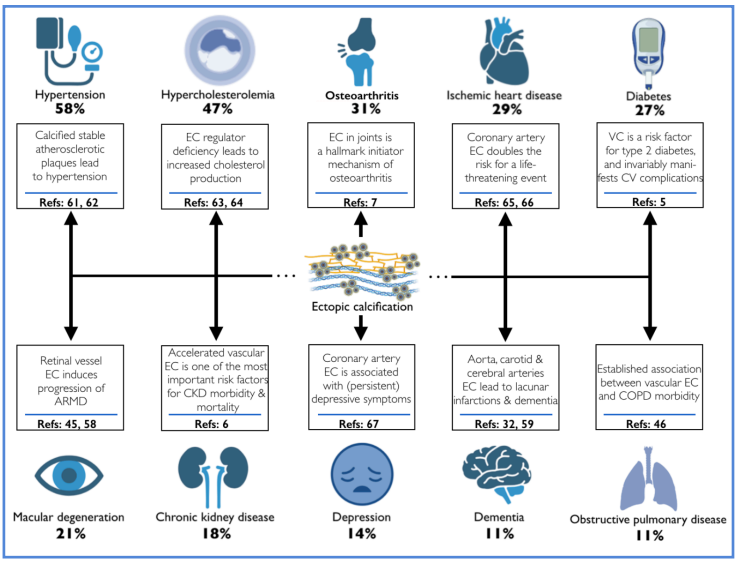
ECTOPIC CALCIFICATION

Ectopic calcification is defined as inappropriate biomineralization occurring in soft tissues. Affecting a wide variety of tissues such as arteries, valves, brain and connective tissues (e.g. skin, joints), EC is highly accelerated in aging, in a wide number of **rare hereditary diseases** as well as in **acquired chronic diseases** such as diabetes mellitus, chronic kidney disease (CKD) and rheumatic diseases.



Demographic changes towards an increasingly **elderly population** result in novel medical challenges, which do not only include improved medical care but also preventive measures and efficacious risk stratification to attain what is referred to as **healthy aging**. Eighty percent of the +65 population has at least one chronic condition, and among the 10 most prevalent ones, EC was shown to be directly **associated to (multi-)morbidity, frailty and mortality**. A common theme is vascular calcification (VC); indeed, aging and several pathologic states, such as obesity, diabetes, or CKD, cause degenerative changes of the vascular walls, including inflammation and VC, leading to arterial stiffening which increases mortality risk three- to fourfold. The impact of VC however extends beyond the CV system; e.g. excess VC of retinal blood vessels and thus the age-related stiffness induces a (fast) evolution of age-related macular degeneration (ARMD). VC also increases the incidence of cerebral lacunar infarctions via decreased diastolic flow and increased pulsatility, leading to symptomatic dementia.

	Hereditary diseases	Acquired diseases
Neurology	COATSplus syndrome; Leuko-encefalopathy with calcifications and cysts; Primary familial brain calcification	Alzheimer disease; Brain tumors; Down syndrome; Lewy body disease; Parkinson disease; Vascular dementia
Cardiology & vascular disease	GACI; PXE; Singleton-Merten syndrome	Heart failure; Valve calcification; Vascular calcification (coronary, PAD); Metabolic syndrome
Endocrinology	Hypophosphatasia; (pseudo)Hypoparathyroidism	Diabetes mellitus; Metabolic syndrome
Nephrology	Gitelman syndrome; PXE	Chronic kidney disease; Nephrocalcinosis, kidney stones
Dermatology	Calcinosis cutis; Calcinosis universalis; Calciphylaxis; PXE; PXE-like syndrome; Tumoral calcinosis	Dermatomyositis; Myositis ossificans; Polymyositis
Rheumatology	Chondrocalcinosis; Hereditary calcification of joints and arteries (ACDC)	CREST syndrome; Osteoarthritis; Scleroderma; Systemic lupus erythematosus
Orthopaedics & physiotherapy	Fibrodysplasia ossificans progressiva; Progressive osseous heteroplasia	Degenerative disease of intervertebral disk; Scoliosis
Ophthalmology	PXE	ARMD
Pneumology	Keutel syndrome	Chronic obstructive lung disease
Generalized	Progeroid syndromes	Sarcoidosis; Systemic sclerosis



Taken everything into account, EC has a significant impact on society; CV disease by itself - the major cause of mortality and morbidity in EU - accounts for 1.9 million deaths and a cost of 210 billion Euro per year. While this highlights the promise of EC as a hitherto overlooked target for risk stratification, diagnosis and preventive intervention, a **comprehensive understanding of the causes of EC is still missing**. The strenght of this ITN is that it will provide a response to the unmet need to understand the mechanisms of EC and that – due to an increasingly elderly population – it will benefit the whole society.

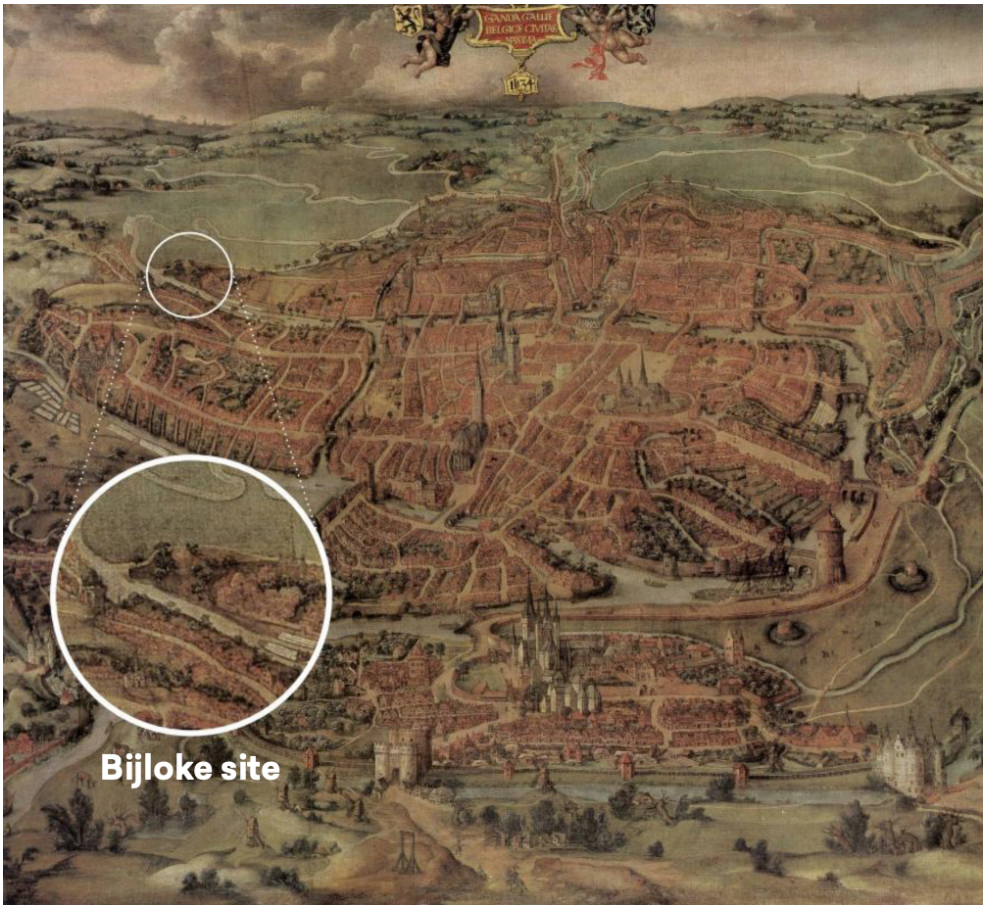
Studies of **rare hereditary EC disorders** have been instrumental in understanding particular aspects of EC pathophysiology, which are anticipated to be applicable also to acquired diseases and aging as well. For example, the hallmark multisystemic EC disorder **pseudoxanthoma elasticum** (PXE), in which we demonstrated accelerated aging processes, has been essential for our understanding of the metabolism of inorganic pyrophosphate (PPi), a calcification inhibitor which is significantly decreased in PXE but also in e.g. CKD patients. In the latter, PPi levels are inversely correlated with CV disease risk and mortality. The liver transporter ABCC6, deficient in PXE, was shown to be the main source of plasma PPi. Further, the retinopathy of PXE bears high similarities with ARMD. Very recently, ABCC6 and systemic PPi were associated with the health of the intervertebral disk and axial skeleton.

Similarly, in **progeria syndromes** characterized by clinical features mimicking physiological aging at an early age, EC occur in at least one-third of patients. They are considered models for human aging and several molecular mechanisms implicated in progeria such as DNA damage response and epigenetic changes are also involved in EC and in physiological aging.

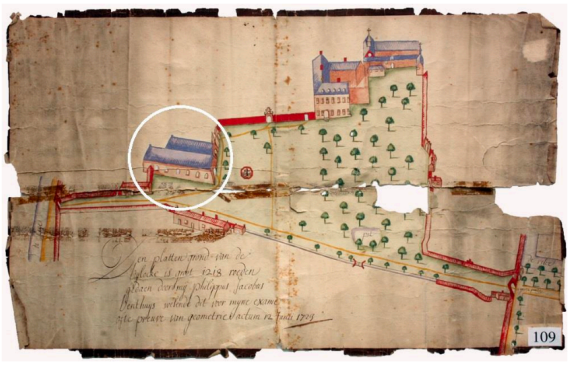
These examples show that hereditary diseases provide a unique opportunity to identify genetic background, pro-and anti-calcifying molecules, metabolic and epigenetic factors that contribute to the development, phenotypic consequences and severity of EC which are translatable to common disorders and natural aging. However, the role of these factors is still only partially uncovered and needs to be clarified. By integrating and expanding our knowledge on these factors, INTEC offers a unique setting for new discoveries and education. The anticipated research, education and dissemination of results will **improve diagnosis and risk stratification** in hereditary and common disorders and as such **contribute to healthy aging** for the general population, based on a deepened understanding of consequences and causes of EC.

OUR VENUE

The Bijloke site is located close to the city centre and has been the heart of healthcare for centuries. Now it is a unique arts and culture campus in the middle of an impressive historic building complex on the banks of the River Leie. The structure and architecture of the buildings - home to one of the best-preserved medieval hospitals in Europe - are the result of more than seven centuries of development in care and medicine.



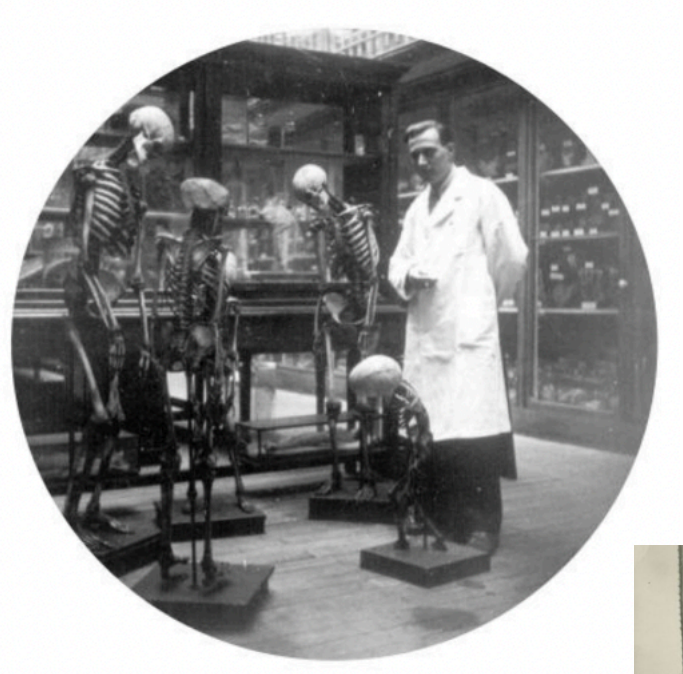
In the **early 13th century**, Ermentrudis ten Hove, member of a well-known Ghent patrician family founded the Maria Hospital in her private home. This small-scale hospital had to disappear when the Dominicans arrived around 1220 and demanded space for their monastery. Around 1250, the construction of a new hospital began, where Cistercian sisters were looking after the sick. The Bijloke was founded on a private or 'enclosed' area, the meaning of the word 'beloken'.

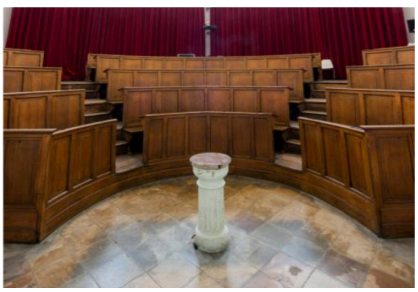


Around 1511, a second, smaller infirmary was built for the seriously ill. It was named the 'Craeckhuys'. 'Craecken' means being seriously ill or dying and may refer to a room where people were nursed before death. Others, however, believe the name may have come from the German 'Krankenhaus', suggesting it was a separate location for the richer citizens of Ghent.

The sisters abandoned the Bijloke in **1797**, as with the annexation of France it became the responsibility of lay people to care for the sick. The situation soon became chaotic and people longed for the sisters to return, which they were allowed to in **1802**. They now worked for the Commission of Civil Hospices, founded by the French Revolutionaries. The 17th century wings of the building were converted into a monastery; the rest housed an old-men's house.

With the **founding of Ghent University in 1817**, the training of doctors and other medical staff was entrusted to the Bijloke Hospital which expanded with classrooms, laboratories, libraries and dissection rooms such as **the Anatomical Theatre** where you will sit for this symposium. This auditorium is part of the Anatomical Institute, designed at the end of the 19th century by Adolphe Pauli for Ghent University. Here, spectators could observe the dissections of human corpses and animal carcasses from the wooden stand. The institute also featured a surgery room and three large laboratories. Students were taught here until 1965 and the people of Ghent visited it with horror.





A school for surgeons and midwives was set up and, between **1863 and 1880**, a new neo-gothic Civil Hospital was built on the site, while in and around the site 4 new institutes of the Faculty of Medicine were constructed.

Mid of the 20th century, when the hospital and Faculty moved to new locations, the city of Ghent purchased the site and restored it to the multifunctional location it is **today**.

VISITING GHENT

Locals and tourists alike love Ghent. What's not to love? We have everything: fascinating art, beautiful architecture and great food. Ghent is a perfect blend of industry and medieval architecture which will appease every travellers European city palette. The nightlife and the cuisine are legendary with some truly great food being served at a range of restaurants that will suit all budgets. Beer is king in Belgium and Ghent is home to the famous Gruut beer.

The history of Ghent begins in the year 630 when St Amandus chose the site of the confluence (or 'Ganda') of the two rivers, the Lys and the Scheldt to construct an abbey. Nearly 1400 years of history are still palpable in the city today: a medieval castle surrounded by a moat, an imposing cathedral, a belfry, three beguinages... Nowhere else does one find so much history per square meter than in the historical heart of Ghent!

PROGRAM

9:00	***Welcome address*** Olivier Vanakker (*INTEC coordinator*)
9:20	**Resolution of inflammation in vascular and valvular calcification** Magnus Bäck (*Karolinska Institutet and Karolinska University Hospital, Sweden*)
9:45	**Different effects of calcification on the chondrocyte in joint diseases** Jessica Bertrand (*Otto-von-Guericke University, Germany*)
10:10	**Tracheal calcification: not so crystal clear!** Hervé Kempf (*University of Nancy, France*)
10:40	***Coffee break***
11:10	**Calcifications in the central nervous system: phenotypes & etiologies** Dimitri Hemelsoet (*Ghent University Hospital, Belgium*)
11:35	**Rare diseases of ectopic calcification in the skin** Ofer Sarig (*Tel Aviv Sourasky Medical Center, Israel*)
12:00	**The ABC of ANKH-oring PPi levels** Flóra Szeri (*Research Center for Natural Sciences, Hungary*)
12:25	***Lunch and poster viewing***
14:00	**Deletion of the P2Y2 receptor aggravates internal elastic lamina calcification in chronic kidney disease mice through upregulation of alkaline phosphatase and lipocalin-2** Britt Opdebeeck (*Antwerp University, Belgium*)
14:15	**More bone without minerals: alternating dietary phosphorus increases bone volume in zebrafish** Silvia Cotti (*Ghent University, Belgium*)
14:30	**The prevalence and characterization of pseudoxanthoma elasticum (PXE) in Finland; a national registry study** Pasi Nevalainen (*Tampere University Hospital, Finland*)
14:45	**Serum Calcification Propensity T50 Associates with Disease Severity in Patients with Pseudoxanthoma Elasticum** Lukas Nollet (*Ghent University Hospital, Belgium*)

15:00 **How much the ABCC6 research can benefit from the protein structures of the artificial intelligence (AI) era?**
Andras Varadi (*Research Center for Natural Sciences, Hungary*)

15:15 ***Coffee break***

15:45 **Effect of lansoprazole on the plasma PPi levels in patients with PXE: a randomized, placebo-controlled, double-blind, crossed-over trial**
Juan Luis Carrillo (*Malaga Virgen de la Victoria University Hospital, Spain*)

16:10 **Treatment options for ectopic calcification diseases**
Yvonne Nitschke (*Münster University Children's Hospital, Germany*)

16:35 **The use of animal models to study ectopic calcification**
Vicky MacRae (*University of Edinburgh, United Kingdom*)

17:00 **Dermal fibroblasts in the calcification process**
Daniela Quaglino (*University of Modena and Reggio Emilia, Italy*)

17:25 ***Wrap up and end of the meeting***

Resolution of inflammation in vascular and valvular calcification

Magnus Bäck

Chronic inflammation is a hallmark of cardiovascular disease and represents a therapeutic target. Importantly systemic inflammation is associated with ectopic cardiovascular calcification. However, anti-inflammation may lead to immunosuppression, hence limiting its implementation in cardiovascular prevention. An optimal immune response must allow a host defence but exclude the development of a chronic response by a timely resolution of inflammation, which is an active process. Lipid mediators represent a key balance to direct the inflammatory trajectory towards either inflammation or resolution. The proinflammatory lipid mediators have been associated with increased soft tissue calcification in atherosclerosis and heart valve disease. The present project specifically studies how proresolving lipid mediators will participate in the resolution of inflammation and a decreased/reversed vascular and valvular calcification processes. Resolvin E1 (RvE1), derived from omega-3 fatty acids, promotes a resolution of inflammation through the G-protein-coupled receptor ChemR23, which is expressed on both immune cells and structural cells in calcified stenotic aortic valves and calcified atherosclerotic lesions. Models with a targeted ChemR23 exhibit increased valvular calcification and more severe aortic valve stenosis, as well as a neutralization of beneficial effects of omega-3 fatty acids. However, the ChemR23 deletion leads to both pro- and anti-calcifying effect in the vascular wall, whereas the RvE1 ligand is consistently protective against vascular calcification both in presence and absence of a stimulated inflammatory response. Taken together, these results implicate a complex interaction between inflammation and ectopic calcification and identify signaling of the omega-3-derived proresolving mediator RvE1 through the ChemR23 receptor as a central anti-calcification pathway, which opens up new avenues for treatment of ectopic cardiovascular calcification.

Different effects of calcium crystals on the joint cells in calcifying joint diseases
Jessica Bertrand

Two types of calcium crystals can be found in the painful knee joint. There are BCP crystals, which have been associated with osteoarthritis (OA), and CPP crystals, which have been associated with chondrocalcinosis (CC). BCP crystals are linked to hypertrophic differentiation of chondrocytes on OA. CPP crystals are somehow associated with chondrocyte senescence. The question is, whether the distinction of crystal types with joint pathologies and effect on the surrounding cells is that simple. The different crystal types in cartilage and synovial membrane of OA and CC patients were identified using RAMAN and SEM-EDX in a cohort of patients and compared with the radiological diagnosis. The cellular phenotype was also analysed by investigating senescence markers in tissue und synovial fluid.

Tracheal calcification: not so crystal clear!
Hervé Kempf

Tracheal cartilage is a C-shaped hyaline cartilage known as permanent cartilage. Compliance and elasticity are the two features required for tracheal cartilage in order to fulfill its function in preventing airway collapse. Tracheal mineralization is a rare condition mostly found in the elderly population where the elasticity of the trachea is compromised leading patients to eventually suffer from dyspnea. In that context, we ought to understand the cellular and molecular mechanisms at the origin of tracheal mineralization that has been unexplored so far and investigate the role of the Matrix Gla Protein (MGP) in the process, as genetic or pharmacological inhibition of this protein has been shown to induce abnormal tracheal mineralization in very young individuals.

Our morphological and histological studies show an unexpected early mineralization of the cartilage rings of the trachea that is detected at only 30 days (P30) after birth in wild-type mice. This mineralization extends in a rostro-caudal pattern, through a process involving terminal differentiation of tracheal chondrocytes. We further demonstrated that, via a similar pattern and comparable mechanism, deficiency of MGP accelerates and expands the overall mineralization of the mouse respiratory tract. Indeed, trachea from *mgp*$^{-/-}$ mice show signs of calcification as early as P14 onwards in cartilage rings but also exhibit an additional and unexpected calcification of their lamina propria, that is not observed in their control littermates.
The present study is the first to comprehensively describe mouse tracheal mineralization and provide evidence that, in the mouse, contrary to the typical notion, tracheal cartilage is not a permanent hyaline cartilage throughout life, as calcification of the cartilage rings appears to be a sudden and early physiological event**.** It also authenticates Matrix Gla protein as a key factor in abnormal mineralization of the tracheobronchial tree.

Calcifications in the central nervous system
Dimitri Hemelsoet

Intracranial calcifications or calcifications in the central nervous system (CNS) generally refer to calcifications within the brain parenchyma or its vasculature. Often CNS calcifications are considered a physiological phenomenon without clinical significance, especially as part of a normal ageing process. Cerebrovascular calcifications are considered to be a risk factor for cerebrovascular complications, especially stroke. Additionally, CNS calcifications occur in systemic diseases (infections, metabolic alterations, toxic causes), vascular malformations, and in an increasing number of genetic disorders. Pathophysiological mechanisms of CNS calcifications are often not well understood and insufficiently or not yet investigated. Postulated pathogenic mechanisms include systemic calcium and phosphate imbalance, mineralizing microangiopathy, blood-brain barrier dysfunction, inflammation, and microgliopathy. A brief review of clinical signs, diagnostic aspects and causes of CNS calcifications will be presented.

Rare diseases of ectopic calcification in the skin
Ofer Sarig

Ectopic calcification refers to the deposition of calcium salts outside the bone tissue. It can be systemic such as in PXE and GACI or prominent in the skin tissue in which case it is referred to as calcinosis. Calcinosis is less discussed and usually less mention in text books, but which can inform us a lot about the management of very common diseases. There are two major forms of **acquired** calcinosis cutis (CC), dystrophic CC (DCC) and metastatic CC (MCC). DCC is the most common cause of calcinosis cutis and is associated with normal calcium and phosphorus levels and results from any form of preceding of injury to the skin as in several autoimmune diseases for example, while MCC is associated with abnormal serum calcium and phosphorus levels, and deposition occurs when calcium phosphate production exceeds a certain value (e.g. chronic renal failure). The inherited counterpart of acquired CC is termed familial tumoral calcinsis (FTC). This is an AR condition, which has been reported prominently in individuals of Middle Eastern and African ancestry, and features the progressive development of calcified masses in skin tissues. Just as there are two major forms of acquired CC, there are two forms of FTC which can be distinguished based on the levels of circulating phosphate. The hyperphosphatemic type of FTC(HFTC) features large masses around the large joints whereas the normophosphatemic subtype of the disease (NFTC) is associated with much smaller masses in an acral location and with significant mucosal involvement. The HFTC subtype of the disease is due to increased renal absorption of Pi leading to hyperPi and subsequent calcium deposition and as such models the metastatic form of **acquired** CC. The NFTC subtype of the disease is secondary to an inflammatory trigger and as such models the dystrophic form of **acquired** CC. Over the past few years, the molecular underpinning of most forms of FTC has been elucidated. We will discuss the genetic basis of inherited CC, the complexity of the current pathogeneic scheme for diseases associated with skin ectopic calcifications and what are the applications we can learn from studying this rare form of inherited FTCs.

The ABC of ANKH-oring PPi levels
Flóra Szeri

The plasma membrane proteins Ankylosis Homologue (ANKH) and the ABC transporter ABCC6 prevent pathological mineralization of joints and soft tissues, respectively, by regulating extracellular levels of the ectopic calcification inhibitor pyrophosphate.
In contrast to the widely accepted view, our recent data clearly demonstrated that ANKH is not a dedicated pyrophosphate exporter but mediates robust cellular efflux of citrate and nucleoside triphosphates, predominantly ATP. Extracellular ATP, generated by either ANKH- or ABCC6-dependent manner, is converted into PPi by ENPP1, the sole extracellular enzyme capable of metabolizing nucleoside triphosphates to pyrophosphate. Our in vitro and in vivo data suggest that the interplay of these three proteins is the critical determinant of the local and systemic pyrophosphate homeostasis in physiological and pathological conditions.

Effect of lansoprazole on the plasma PPi levels in patients with PXE: a randomized, placebo-controlled, double-blind, crossed-over trial
Juan Luis Carrillo

Pseudoxanthoma Elasticum (PXE) is a rare disease characterized by calcification of elastic fibers due to a reduction in inorganic pyrophosphate (PPi) plasma levels, a natural product that inhibits the formation of hydroxyapatite and the calcification in tissues. The PPi levels are regulated by two enzymes: tissue-nonspecific alkaline phosphatase (TNAP), which transforms PPi in two molecules of Pi and the ectonucleotide pyrophosphatase/phosphodiesterase (ENPP1) able to produce PPi from ATP. Lansoprazole is a proton-pump inhibitor licensed to treat diseases related to the gastric acid output that has been shown to inhibit partially the effect of TNAP.

We conduct a double-blind, randomized, placebo-controlled and crossed-over clinical trial The aim of this study was to test in a clinical trial the effect of Lansoprazole for 8 weeks on plasma PPi levels in subjects with PXE.

Treatment options for ectopic calcification diseases
Yvonne Nitschke

Pathological soft tissue calcification can occur during aging, in several common conditions such as diabetes, hypercholesterolemia, and renal failure and in certain genetic disorders. Although the pathomechanisms of pathologic calcification are poorly understood, major progress has been made in recent years in defining the underlying genetic defects in inherited disorders of ectopic calcification. Three monogenic disorders of pathologic calcification, namely pseudoxanthoma elasticum (PXE), generalized arterial calcification of infancy (GACI), and calcification of joints and arteries (CALJA) are caused by an altered purine and pyrophosphate (PP_i) metabolism leading to reduced circulating levels of PP_i. PP_i is the principal physiologic inhibitor of calcium hydroxyapatite deposition in soft connective tissues. Currently, most therapies focus on alleviating the symptoms of these diseases. However, extensive studies have led to several new approaches to treat PXE, GACI and CALJA. This presentation will summarize therapeutic strategies targeting different players in purine and pyrophosphate metabolism for direct inhibition of calcification by supplementation with various substances.

The use of animal models to study ectopic calcification
Vicky MacRae

Ectopic calcification contributes to high morbidity and mortality, and is a disease with multi-faceted contributing factors in an actively regulated process. Due to the complex pathophysiology, various research models exist evaluating different aspects of

calcification. I will aim to give an overview of the key animal models used to study the molecular processes of ectopic calcification, with a focus on transgenic rodents.

Dermal fibroblasts in the calcification process
Daniella Quaglino

Fibroblasts are typical mesenchymal cells of soft connective tissues and have been widely investigated in the repair and in the fibrotic processes; however, recent data highlighted their role also in ectopic calcification. Since dermal fibroblasts can be easily obtained from small tissue samples, are quite stable in culture for several passages and can respond to a variety of exogenous stimuli, they represent a useful and interesting in vitro model to investigate the mechanisms involved in hydroxyapatite deposition.
Under specific culture conditions, fibroblasts produce a calcified matrix whose deposition can be modulated by molecules inhibiting (e.g., TNAP inhibitors) or promoting (e.g., platelet lysate) the mineralization process. Furthermore, fibroblasts can also represent a suitable in vitro model to explore cell behavior, protein expression and signaling pathways in aging (e.g., in vitro and ex-vivo aging) and in pathologic conditions (e.g., fibroblasts from Pseudoxanthoma elasticum affected patients). Transcriptomic, proteomic, biochemical, immunocytochemical and morphological analyses of dermal fibroblasts in different experimental conditions can be therefore effectively applied to integrate data addressing the complexity of the calcification process.

Deletion of the $P2Y_2$ receptor aggravates internal elastic lamina calcification in chronic kidney disease mice through upregulation of alkaline phosphatase and lipocalin-2

Britt Opdebeeck[1], Ine Huysmans[1], Astrid Van den Branden[1], Isabel R Orriss[2], Patrick C D'Haese[1] and Anja Verhulst[1]

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Calcification of the medial layer, inducing arterial stiffness, contributes significantly to the cardiovascular mortality in patients with chronic kidney disease (CKD). Extracellular nucleotides block mineralization of arteries by binding to purinergic receptors including the $P2Y_2$ receptor. This study investigates whether a deletion of the $P2Y_2$ receptor influences the development of arterial media calcification in CKD mice. Animals were divided in (i) wild type mice with normal renal function (control diet) (n = 8), (ii) $P2Y_2R^{-/-}$ mice with normal renal function (n = 8), (iii) wild type mice with CKD (n = 27) and (iv) $P2Y_2R^{-/-}$ mice with CKD (n = 22). To induce CKD, animals received an alternating (0.2-0.3%) adenine diet for seven weeks. All CKD groups developed a similar degree of chronic renal failure as reflected by high serum creatinine and phosphorus levels. Also, the presence of CKD induced calcification in the heart and medial layer of the aortic wall. However, deletion of the $P2Y_2$ receptor makes CKD mice more susceptible for the development of calcification in the heart and aorta (aortic calcium scores, CKD-wild type: 4.1±7.3 mg calcium/g wet tissue and CKD-$P2Y_2R^{-/-}$: 11.5±17.2 mg calcium/g wet tissue). Based on serum and aortic mRNA markers, this $P2Y_2R^{-/-}$ mediated rise in CKD related arterial media calcification could be associated to an elevation of calcification stimulators, including alkaline phosphatase and inflammatory molecules interleukin-6 and lipocalin 2. The $P2Y_2$ receptor should be considered as an interesting therapeutic target for tackling CKD related arterial media calcification.

More bone without minerals: alternating dietary phosphorus increases bone volume in zebrafish

Silvia Cotti[1,2], Ann Huysseune[1], Antonella Forlino[2] and P. Eckhard Witten[1]

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Background
Dietary phosphorus (P) is essential for mineralisation of the collagen type I based bone matrix. Low-P dietary intake results in reduced bone mineralisation, but increased formation of non-mineralised bone matrix in zebrafish[1] and salmon[2,3]. We test the mineralisation potential of the non-mineralised bone matrix formed in low-P fed zebrafish, by providing the animals with adequate dietary P. The aim is to mineralise the formerly non-mineralised matrix and to increase the volume of mineralised bone.

Methods
WT zebrafish (1 month old) were fed a low-P diet (0.5% total P, LP) for 8 weeks. Subsequently, zebrafish continued for 6 weeks on a LP, regular-P (1.0% total P, RP) or high-P (1.5% total P, HP) diet. Controls received RP diet throughout. Alizarin red, X-rays, micro-CT and several histological techniques were used to analyse bone phenotype and mineralisation.

Results
Zebrafish with LP history have increased total bone area at vertebral endplates compared to controls ($p<0.001$). Likewise, a dramatic increase in the volume of vertebral centra bone (mineralised and non-mineralised) is observed in all fish with LP history. The non-mineralised matrix formed during the LP period eventually mineralises in LP-RP and LP-HP fish, the mineralisation extent depends on dietary P intake. Bone mineral density (BMD) is reduced in all groups with LP history compared to controls ($p<0.001$), but BMD resumes control values when increasing dietary P. All fish have vertebral centra without malformations and intact intervertebral spaces.

Discussion
The low-P dietary intake stimulates the formation of a large non-mineralised bone volume that retains the ability to mineralise when adequate dietary P is provided. The result is a dramatic increase of healthy mineralised bone volume. Different from human diseases with pathological increased bone mass, no skeletal malformations occur in zebrafish.

Conclusion
This zebrafish model shows that bone formation and mineralisation are independent processes. Our protocol has already been applied to partly rescue the *Chihuahua* zebrafish osteogenesis imperfecta model[4]. Low-P conditions have the potential to mitigate bone loss, e.g. caused by osteoporosis or aging.

Funding
EU-H2020-MSCA-766347, BOF.ITN.2021.0012.001.

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Title: The prevalence and characterization of pseudoxanthoma elasticum (PXE) in Finland; a national registry study

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Background: Pseudoxanthoma elasticum (PXE, OMIM#264800) is an inborn error of metabolism caused by plasma pyrophosphate deficiency resulting from biallelic pathogenic variants of the adenosine triphosphate-binding cassette C6 (ABCC6) gene. Main phenotype is that of ectopic soft tissue calcification causing typical pseudoxanthomas of the skin folding areas; arteriosclerosis causing peripheral obstructive arterial disease; and visual loss by complications of the calcified retinal Bruch's membrane in adult age. Other manifestations include nephrolithiasis, gastrointestinal hemorrhages and strokes.

Methods: Our aim was to evaluate and characterize the Finnish PXE population. We did a nationwide registry search to identify patients with ICD-10 code Q82.84. All life-long medical records available from hospitals and health care centers were gathered. We excluded misdiagnosed cases and cases with insufficient data.

Results: The prevalence of PXE is 1:260 000 in Finland. Mean age at diagnosis was 33 years (range 10-63 years). Visual and vascular complications occur more frequently when conventional cardiovascular risks are present. More than half of the patients (57%) had received intra-vitreal vascular endothelial growth factor inhibitor injections. Every fifth patient (19%) in the PXE population had at least one vascular malformation. Nephrolithiasis was found in 19%, cerebrovascular arterial disease in 24%, peripheral arterial disease in 29% and intra-abdominal hemorrhagic events in 29% of patients. A third (33%) of patients had the common homozygous c.3421C>T, p.Arg1141Ter variant as the ABCC6 genotype and nine other homozygous or compound heterozygote allelic variants were found.

Conclusion / Discussion: The prevalence of diagnosed PXE is lower in Finland compared with other countries. Impaired visual acuity is the most prevalent and meaningful complication. Various vascular malformations may be a previously unrecognized feature of PXE.

Serum Calcification Propensity T50 Associates with Disease Severity in Patients with Pseudoxanthoma Elasticum

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Pseudoxanthoma elasticum (PXE) is a currently intractable genetic disorder characterized by progressive ectopic calcification in the skin, eyes and arteries. Therapeutic trials in PXE are severely hampered by the lack of reliable biomarkers. Serum calcification propensity T50 is a blood test measuring the functional anticalcifying buffer capacity of serum. Here, we evaluated T50 in PXE patients aiming to investigate its determinants and suitability as a potential biomarker for disease severity. Fifty-seven PXE patients were included in this cross-sectional study, and demographic, clinical, imaging and biochemical data were collected from medical health records. PXE severity was assessed using Phenodex scores. T50 was measured using a validated, nephelometry-based assay. Multivariate models were then created to investigate T50 determinants and associations with disease severity. In short, the mean age of patients was 45.2 years, 68.4% was female and mean serum T50 was 347 min. Multivariate regression analysis identified serum fetuin-A ($p < 0.001$), phosphorus ($p = 0.007$) and magnesium levels ($p = 0.034$) as significant determinants of T50, while no correlations were identified with serum calcium, eGFR, plasma PPi levels or the *ABCC6* genotype. After correction for covariates, T50 was found to be an independent determinant of ocular ($p = 0.013$), vascular ($p = 0.013$) and overall disease severity ($p = 0.016$) in PXE. To conclude, shorter serum T50 - indicative of a higher calcification propensity - was associated with a more severe phenotype in PXE patients. This study indicates for the first time that serum T50 might be a clinically relevant biomarker in PXE and may thus be of importance to future therapeutic trials.

How much the ABCC6 research can benefit from the protein structures of the artificial intelligence (AI) era?

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ABCC6 is the disease gene of pseudoxanthoma elasticum, a rare calcification disorder. Its translated polypeptide chain is ABCC6, a transmembrane (TM) protein which is supposed to function as an ATP-transporter and plays an essential role in regulating ectopic calcification.

TM proteins are major drug targets, but their structure determination, a prerequisite for rational drug design and mechanistic studies at molecular level, remains challenging. DeepMind's AlphaFold2 (AF2) is an AI approach for predicting protein structure from sequence and appears to be the holy grail of structural biology. AF2 provided a large number of protein structures with high quality in the past year greatly expanding the structural coverage of the sequence space. Our test on the AF2-generated structures of a large number of ABC-proteins suggested that AlphaFold2 performs well in the case of these large TM proteins and its neural network is not overfitted. First, helix packing in AF2-predicted ABC models overlapped with experimental folds, what gives credential to AF2-structures of proteins not enjoying experimentally determined 3D structure (eg. ABCC6). Moreover, AF2 is likely able to highlight novel structural features, since it correctly predicted an ABC TM fold, the structure of MlaE, which was not included in its training set.

We focus in our ABCC6 studies to the following:

- The structural elements supposed to be involved in substrate binding;
- The location of the extracellular epitope;
- Disease-causing missense mutations affecting structural elements of domain-domain interface regions,
- The unique feature of long ABCC proteins, since ABCC6 falls into this group. These proteins possess a unique domain of five transmembrane helices (TMD0) at their N-terminus, forming a domain topology of TMD0-L0-TMD1-ABC1-TMD2-ABC2. Very little is known about the structure and function of TMD0.

Based on our results, we conclude that cautious applications of AlphaFold2 predicted atomic coordinates of ABC protein, including the ABCC6 structural model, will advance ABC structure related studies at an unexpected level.

Lysyl oxidases (LOX(L)) promote pathologic chondrocyte calcification

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Pathologic calcification (PC) is the deposition of calcium-containing crystals in soft tissues. PC of cartilage is a hallmark of osteoarthritis (OA), a degenerative joint disease characterized by cartilage degradation. We highlighted here that chondrocyte calcification is crucially associated with lysyl oxidase-like (LOX(L)) enzymes, catalyzing cross-linking formation of collagen fibrils in the extracellular matrix (ECM). On the one hand, calcification medium (CM) and calcium containing crystals induced LOX(L) activity in chondrocytes *in vitro*. On the other hand, treatment of calcifying chondrocytes (chondrocytes cultures in CM) with the pan-LOX(L) inhibitor BAPN decreased crystals production in both monolayer and 3D culture, through inhibition of both gene expression of *Anx5*, *PC1*, *Pit-1/2* and Alp activity. We identified that the pro-calcifying effects of LOX(L) were mediated via their classical cross-linking activity, as diminished cross-links in pre-formed extracellular matrix paralleled diminished chondrocyte calcification. Importantly, BAPN reverted the hypertrophic phenotype of calcifying chondrocytes, reducing expression of *Runx2* and *Col10* and increasing that of early differentiation *Sox9* and *Col2*. In the same conditions, LOX(L) inhibition downregulated *Col1* and *Col3*, whose uncontrolled production can lead to ECM fibrosis and calcification. BAPN also inhibited the expression and release of pro-calcifying cytokine Il-6, while also reducing mitochondrial and cytoplasmic ROS, known to be associated with PC. Additionally, we revealed in calcifying chondrocytes that BAPN inhibition decreased cartilage catabolic enzymes (*Mmp3*, *Mmp13* and *Adamts5*). Finally, in the murine OA model based on menisectomy, we found that BAPN administration (starting from the surgery day) led to decreased knee joint calcifications as measured by CT-scan performed two month after surgery. Collectively, our results revealed for the first time a pro-calcifying effect of LOX(L) in chondrocytes, through classical (cross-linking of extracellular matrix) and non-classical pathways (i.e. chondrocyte hypertrophy, increased matrix synthesis and degradation, and exacerbated IL-6 and ROS production). In conclusion, LOX(L) provide a novel target for future therapies aimed at treating pathologic cartilage calcification.

Oral PPi treatment in Systemic Sclerosis: absorption kinetics and safety

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Systemic sclerosis (SSc) is a progressive connective tissue disease resulting in damage to multiple organs. An estimated 18-49% of patients develop calcinosis, dystrophic calcification affecting skin and subcutaneous soft tissue, most frequently in the fingers and hands. Mineralized deposits consist predominantly of hydroxyapatite (HAP).
Cutaneous calcinosis lesions are typically painful, and cause a significant burden for the affected patients. The pathomechanism of the disease is poorly understood, precisely how and why SSc leads to ectopic calcification in a subset of SSc patients remains to be elucidated. Currently there is no universally effective treatment for SSc, or calcinosis in SSc. Inorganic pyrophosphate (PPi) is a potent natural inhibitor of HAP crystal growth. Plasma PPi concentrations were found to be lower in systemic sclerosis patients than in age- and sex-matched healthy control subjects in our cohort of Hungarian patients recruited at the University clinics of Szeged and Debrecen (1.4±0.3 vs. 1.7±0.3 μM), and similar observations have been made in a US cohort of SSc patients.
Based on these results, a clinical trial to test the effect of oral PP i treatment was initiated, with the participation of SSc patients with calcinosis symptoms from three university clinics in Hungary. We present phase I absorption kinetics and safety data of oral pyrophosphate treatment in 10 SSc patients taking a single oral dose of 50 mg/kg disodium salt of pyrophosphate, and in an additional 10 patients who received a lower dose of 25 mg/kg, but continued taking it for seven days.

Developing a mouse model to investigate diabetes-related vascular calcification and the role of ABCC6

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Cardiovascular calcification is commonly associated with diabetes mellitus (DM) and diabetic kidney disease (DKD), and is a leading cause of death in diabetic patients. Mutations in the gene encoding the ABCC6 transporter result in similar cardiovascular calcification as observed in diabetic patients (without other diabetic symptoms). The protective role of ABCC6 in soft tissue calcification is thought to be due to its role in controlling plasma pyrophosphate (PPi) level.

In order to evaluate the molecular mechanisms underlying the pathological calcification symptoms in DM and DKD and to clarify the potential role of ABCC6 in these processes, we chemically induced Type I diabetes in wt, *Abcc6+/-* and *Abcc6-/-* mice. Our preliminary results show that diabetic mice have hyperglicemia, increased plasma urea and creatinine, and decreased plasma albumin levels, as expected. Diabetic *Abcc6-/-* mice develop more pronounced vascular calcification compared to *Abcc6-/-* controls. We also found that PPi levels both in plasma and in urine of diabetic mice are decreased, while plasma ALP activity increased. We could observe decreased plasma PPi level also in human diabteic patiens.

We are currently investigating the efficacy of PPi treatment in the prevention of cardiovascular calcification developing under diabetic conditions.

This mouse model provides an excellent tool to investigate/discover important key regulators of diabetes-related vascular calcification, and therefore may help to find new targets for the prevention of vascular calcification and early biomarkers in the progression of the disease. The contribution of ABCC6 is particularly important as the estimated frequency of heterozygous carriers of ABCC6 mutations in the general population is 1 in 80.

ISSEC: An International Scientific Society of Ectopic Calcification dedicated to the advance of knowledge and awareness for ectopic calcification diseases.

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The International Society of Ectopic Calcification (ISSEC) was recently created following the successful COST Action 'EuroSoftCalcNet' that ended in 2021. Its main aim is to facilitate contacts between all actors dealing with ectopic calcification (EC).

EC is defined as inappropriate biomineralization in soft tissues. Affecting a large variety of tissues such as arteries, brain and connective tissues, EC is accelerated in aging, in a wide number of hereditary diseases as well as in acquired diseases such as chronic kidney disease (CKD).

EC diseases often have an unpredictable evolution and are still largely incurable because of the partially uncovered mechanisms underlying EC and the interindividual variability. Furthermore, there is limited awareness for EC in the medical community and among the general population.

Studies of rare hereditary EC disorders are instrumental in understanding many aspects of EC pathophysiology. For example, the hallmark of a multisystemic EC rare disorder is Pseudoxanthoma Elasticum (PXE), which was essential for our understanding of the inorganic pyrophosphate (PPi) metabolism, a calcification inhibitor that is significantly decreased in PXE but also in CKD. In the latter, PPi levels are inversely correlated with cardiovascular disease risk and mortality. The liver transporter ABCC6, deficient in PXE, was shown to be the main source of plasma PPi.

This example shows that hereditary diseases provide a unique opportunity to identify genetic background, pro-and anti-calcifying molecules, metabolic and epigenetic factors that contribute to the development, phenotypic consequences and severity of EC which are translatable to common disorders and natural aging. However, the role of these factors is still only partially uncovered.

By joining together all stakeholders - patients and their families, clinicians, researchers - ISSEC aims to foster new discoveries to better understand, manage and treat EC disorders and to create worldwide awareness for the EC burden and to improve EC patients' position.

The Abcc6a Knockout Zebrafish Model as a Novel Tool for Drug Screening for Pseudoxanthoma Elasticum

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Background/Objectives:

Pseudoxanthoma elasticum (PXE) is a currently intractable ectopic mineralization (EM) disorder due to bi-allelic ABCC6 mutations. PXE patients have multisystemic EM, while heterozygous carriers present mainly cardiovascular EM. Rapid, cost-effective discovery of therapeutic drugs can be done by compound screening in zebrafish, but this approach is unvalidated in PXE. We validated a stable CRISPR/Cas9 *abcc6a* knockout zebrafish model – which has spinal column hypermineralization as primary phenotypic feature – as a model system for compound screening in EM.

Methods:

We evaluated the anti-mineralization potential of five compounds, which has (anecdotal) positive effects reported in *Abcc6-/-* mice or PXE patients. *Abcc6a-/-* zebrafish larvae were treated from 3 to 10 days post-fertilization with vitamin K1, sodium thiosulfate (STS), etidronate, alendronate or magnesium citrate and compared to untreated fish. Following alizarin red staining, alterations in spinal hypermineralization were semiquantified.

Results:

Vitamin K1 (80µM), etidronate (100µM) and alendronate (100µM) reduce hypermineralization by 42% , 33% and 39% respectively compared to untreated fish ($P<0.05$). We show for the first time in a PXE model that 20µM STS reduces mineralization by 55%, but higher doses paradoxically result in EM. Magnesium citrate (10mM) reduces mineralization by 45% and, as only compound, also has an anti- mineralizing effect in heterozygous zebrafish (hypermineralization reduction of 77%). Physiological bone mineralization was not affected by any of the compound screens.

Conclusion:

We demonstrate that the use of our *abcc6a-/-* zebrafish model is a promising strategy for drug discovery against EM.

The use of *Acomys cahirinus* to study the role of Matrix Gla Protein in tissue regeneration

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Matrix Gla protein (MGP) is a vitamin K-dependent protein, involved in vascular calcification, preventing the trans-differentiation of vascular smooth muscle cells into osteoblasts, and acts as an inhibitor of cartilage calcification. Described MGP functions also include regulating cell differentiation and angiogenesis. Mutations in MGP cause a rare autosomal recessive genetic disorder called Keutel syndrome (KS) whose patient's major traits include abnormal calcification of cartilaginous tissues resulting in or associated with malformations of skeletal tissues and cardiovascular. An increased expression of MGP was also observed in calcinotic skin of patients with systemic sclerosis. MGP appears to be involved in the development and progression of various human tumors, however with different expression patterns depending on the location and type of tumor. Furthermore, it was suggested that MGP might be used for designing novel inhibitors that can promote muscle regeneration or treat muscle atrophy.
Given that MGP could be involved in different systems' pathophysiology including cell differentiation in various contexts, we investigated if MGP could be involved in the regeneration process. The objective of this study was to evaluate the possible role of MGP during the differentiation of newly formed tissues.
The ability to regenerate damaged or missing organs has long been considered a primordial objective of modern medicine. This capacity is present in almost all metazoans. Among vertebrates, it has been reported regeneration in urodele amphibians and, to a lower extent, in teleost fish that can also regenerate several structures. In mammals, on the other hand, tissue regeneration is rarely observed, since response to injury occurs mainly by wound healing through fibrotic scarring. Recently, the African spiny mouse (*Acomys cahirinus*) has arisen as an emerging model of mammalian epimorphic regeneration. This animal is capable of non-fibrotic regeneration of extensive dermal wounds, including dermis, epidermis, hair follicles, sebaceous glands and adipose tissue.
In this work, we observed that in *A. cahirinus*, the MGP protein structure is very similar to the human homologue, containing the same structural domains. During *A. cahirinus* ear regeneration, *MGP* is initially down-regulated, but in the latest stages of cell differentiation, we observed an increase in *MGP* expression suggesting that it is involved in ear regeneration. MGP protein localization during the late stages of regeneration showed its expression in the epidermis, hair follicles, cartilage, and in the muscle cells situated near the dorsal side of the cartilage. Altogether, these data suggest a possible role of MGP in the later stages of regeneration, namely during cell differentiation of newly formed tissues.

Title: Correlation of clinical fundus images with molecular and histopathological analysis of calcified Bruch's membrane in patients with pseudoxanthoma elasticum

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Purpose: Pseudoxanthoma elasticum (PXE) is a rare genetic disorder leading to ectopic calcification of Bruch's membrane (BrM). This can result in reduced metabolic exchange between the retina and choroid, atrophy of retinal pigment epithelium, and formation of angioid streaks with choroidal neovascularization, all of which can lead to vision loss. Here, a histopathological study investigating the composition and distribution of calcified Bruch's membrane in eyes with PXE followed by correlation with clinical fundus images is presented.

Materials and Methods: Six cadaveric eyes that were clinically diagnosed with PXE were obtained from the Netherlands Institute for Neuroscience, Amsterdam, or the Dutch National Expertise Center for PXE, Utrecht. Eyes were either flat-mounted on to glass slides or embedded in paraffin wax and sectioned (7 µm). To visualize the distribution of calcified BrM, flat mounted and cross-sectioned tissues were stained with OsteoSense 680EX, a fluorescent dye specific for the inorganic calcium phosphate, hydroxyapatite. The elemental composition of BrM was analysed using energy dispersive x-ray spectroscopy (EDX) whilst time of flight-secondary ion mass spectrometry (TOF-SIMS) was used for mineral composition analysis.

Results: The distribution BrM calcification changes across the central-far peripheral axis in eyes with PXE. A meshwork-like pattern of calcification was observed in the centra and mid peripheral regions of flat mounted PXE eyes, with a marked reduction in staining in the far-periphery. The stained cross-sections showed an extensive and confluent layer of calcified BrM in the central region with intermittent staining in the far periphery. EDX confirmed the enrichment of calcium and phosphorus in BrM of PXE eyes with elemental mapping of these elements showing a distribution similar OsteoSense staining. TOF-SIMS confirmed that some calcifications in PXE eyes were formed of hydroxyapatite. The distribution of BrM calcification appeared to correlate with findings on prior imaging in one patient

Conclusions:

Extensive calcification is present in the BrM of eyes from patients with PXE. Confluent calcification is observed in the central region with intermittent calcification present towards the far periphery. Correlation of histopathology and elemental/molecular analyses with clinical images suggests that the phenotype observed on fundus imaging modalities is likely associated with the distribution of calcification in the BrM.

NOTES

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