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ECTOPIC MINERALIZATION RESEARCH GHENT

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A digital version of the thesis can be accessed through the following link:

http://bit.ly/3DHiK3T

FACULTY OF MEDICINE AND HEALTH SCIENCES

Revisiting the molecular basis for ectopic mineralization, using pseudoxanthoma elasticum as a model

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Summary

Ectopic mineralization (EM) refers to the pathological deposition of calcium phosphate crystals in soft tissues, where such calcification does not normally occur. This can lead to significant morbidity and mortality due to the resulting disruption of normal tissue function. EM is a hallmark of several prevalent disorders, such as atherosclerosis, calcific aortic valve disease, and osteoarthritis. Understanding the molecular mechanisms behind this aberrant process is crucial for developing targeted therapies and improving patient outcomes. Research on rare, heritable disorders can reveal common pathomechanisms, offering broader implications for understanding and potentially treating more prevalent diseases.

Pseudoxanthoma elasticum (PXE) serves as an exemplary model for studying EM disorders due to its defined genetic basis and clinical manifestations. Biallelic loss-of-function variants in the ***ABCC6*** or the ***ENPP1*** gene are responsible for the progressive calcification of elastic fibers, which manifests in the skin, eyes, arteries, and other internal organs. Biallelic variants in *ENPP1* or *ABCC6* can also cause a disorder at the severe end of the clinical spectrum of EM, called generalized arterial calcification of infancy (GACI).

A significant subset of PXE patients has an unresolved genotype because causal genetic variants may be missed during routine diagnostic screening or because the pathogenicity of identified variants remains unclear. Furthermore, disease severity varies widely among individuals, making it challenging to predict disease progression due to its slow and progressive nature. To date, no clear correlations between genotype and phenotype have been established.

In this doctoral research project, we aimed to address these issues by (i) developing reliable variant detection strategies for PXE, (ii) developing a standardized approach for variant interpretation, and (iii) identifying a correlation between genotype and phenotype.

In **paper 1**, we developed a strategy that allows for the reliable detection of variants in the first nine exons of the *ABCC6* gene, a region that is highly homologous to the *ABCC6P1* and *ABCC6P2* pseudogenes. In **paper 2**, we highlighted the importance of deletion/duplication analysis in the *ABCC6* and *ENPP1* genes in both PXE and GACI patients. To this end, we used a custom micro-array-based comparative genomic hybridization (aCGH) approach to detect structural *ENPP1* variants and expanded the *ABCC6* and *ENPP1* mutation spectrum with novel structural variants.

In **paper 3**, we aimed to improve variant interpretation and reduce interpretation discrepancies by developing a standardized approach for the classification of *ABCC6* variants. We based our approach on the Sherloc classification system, which provides a semiquantitative framework, and tailored it to the genetic architecture of PXE. This classification approach was then used in collaborations with other research groups to aid in the characterization of the Finnish PXE population (**paper 4**) and of *ABCC6* variants that display incomplete penetrance (**paper 5**).

Since most detected variants in the *ABCC6* gene are missense variants for which the clinical significance remains unclear, we aimed to create a hepatocyte-like cell line model that would allow for high-throughput functional analysis. For this, we used CRISPR/Cas9 technology to genetically ablate the *ABCC6* gene in the HepG2 cell line, but we were unable to fully characterize ABCC6 activity due to several technical challenges. This reflects the inherent difficulty of functional characterization of genetic variants in an *in vitro* setting that mimics the main expression site of ABCC6.

In **paper 6**, we collaborated on a genotype-phenotype correlation study of a large Dutch PXE cohort, which showed that PXE patients with two truncating *ABCC6* variants tend to develop retinal complications earlier and have more arterial calcification, as measured by (semi)quantifiable markers.

In conclusion, we demonstrate that molecular analysis of PXE genes necessitates specific adaptations to ensure reliable variant detection. Firstly, pseudogene interference can be mitigated through gene-specific enrichment and a masked mapping strategy in next- generation sequencing. Secondly, the gold standard for copy number variant analysis, MLPA, can be effectively complemented with custom aCGH analysis to identify or confirm structural variants in PXE-associated genes. Furthermore, we have developed a systematic framework for the interpretation of *ABCC6* missense variants using established classification guidelines, which can be utilized in demographic studies and genotype-phenotype correlations.

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