University of Washington
Department of Bioengineering

Qualifying Examination

A Quantitative Systems Approach for Studying and Treating Arterial Calcification Due to CD73 Deficiency

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1 Abstract and Specific Aims

Vascular calcification in both the intima and media of vessels is associated with increase risk for cardiac events and mortality. Given the significant clinical impact of arterial calcification, the mechanism and genetic basis behind its clinical presentation has been a subject of intense study.

Recently, the human gene *NT53* that encodes CD73, the enzyme responsible for converting extracellular AMP to adenosine, has been implicated as a key component behind the metabolic pathway for inhibiting medial vascular calcification. Individuals with mutations in NT5E result in a disease phenotype of arterial calcification and distal joint calcification (ACDC). As of the writing of this proposal, there is no standard treatment or therapy for alleviating this condition.

This study proposes the development of a quantitative systems model, built alongside and informed through *in vitro* experimentation, for elucidating the mechanism behind the ACDC phenotype. This model will enable in-depth investigation of the medial vascular calcification pathway, consolidation and validation of the mechanistic understanding of the disease, and identification and prediction of efficacious new therapeutic interventions, which will be confirmed experimentally. After establishing a predictive *in silico* model of the biological mechanism behind ACDC, sensitivity and flux balance analysis will be used to identify targets within the pathway for therapy, which will be confirmed in a murine disease model. The general approach in building this mechanistic model is iterative and hypothesis driven. Experimental results will serve to tune and build confidence in the proposed mechanisms, leading to better understanding of the impact due to CD73-deficiency and potential treatments for the condition. While beyond the scope of this proposal, the systems modeling approach introduced here may also prove useful for studying a number of related diseases stemming from defects within biomolecular components upstream and down stream of CD73.

1.1 Specific Aim 1: Develop *In Vitro* Platform for Exploring ACDC Pathway.

An *in vitro* model consisting of vascular smooth muscle cells, will serve as a platform for running molecular biology experiments for mechanistic exploration and model building. Through review of the current literature, several surface bound enzymes, primarily ENPP1 and TNAP, were identified as major components contributing to pyrophosphate depletion, leading to mineralization of the arterial wall, and will also be incorporated in the initial mechanistic hypothesis. Antibodies will be developed to specifically to bind to each of the surface proteins of interest, allowing for labeling and quantification of the enzymes, in addition to serving as a method for inhibiting specific reactions. This aim will achieve quantification and control over the system that will be modeled.

1.2 Specific Aim 2: Build Quantitative Systems Model of Medial Arterial Calcification.

A system of coupled differential equations will be used to mathematically model the kinetics of the arterial calcification system. The biomolecular interrogation techniques from Specific Aim 1 will be used experimentally fit parameters within the model. Enzyme surface expression levels and trafficking will be determined, as it is important from a modeling perspective to quantify the total available enzyme levels. Endogenous metabolite generation and degradation rates will be measured under full blockage of the pathway. The kinetics of each enzyme in the system will be measured in insolation by selectively blocking the activity of other enzymes in the pathway. Parameters will be fit through standard nonlinear regression techniques and the model will be built in steps through the incremental coupling of the system components. Once the full model has been established, uncertainty analysis will be performed to determine how uncertainty in the model parameters propagates to uncertainty in the model predictions.

1.3 Specific Aim 3: Investigate Potential Therapeutic Interventions for Treating CD73 Deficiency *In Vitro* and *In Silico* Along with Extrapolating the Results for *In Vivo* Study in an Established Murine Model.

An *in vitro* and *in silico* disease model will be produced through antibody blocking of CD73 in cultured cells and removing the CD73 contribution in the mathematical model. Sensitivity and flux balance analysis will be used to determine which other parts of the network would be suitable therapeutic targets for restarting PPi/Pi balance. An animal model of disease, CD73 -/- mice, will be used to perform a test of principle for phosphate balance recovery. Potential therapies include, but is not limited to, bisphosphonates (a PPi analog), adenosine anaologes, lansoprazole (inhibitor of TNAP), and dipyridamole (adenosine signaling inhibitor). Therapy selection will be assisted by *in vitro* and model simulation results.

2 Background and Significance

2.1 Vascular Calcification

Vascular calcification is the process in which hydroxyapatite mineral deposits are formed in the walls of blood vessels. Arterial calcification is a well-defined risk factor in significantly increased patient mortality. [Shaw et al. (2003); @Chiu2010; @Blacher2001; @London2003] While once thought to be a passive process of deposition due to elevated electrolyte imbalances in the blood, vessel calcification has been discovered to be an active process that is similar to bone formation and remodeling. (Boström et al. 1993)

Calcification can occur in either the media (within vessel walls) or intima (vessel lumen interior). Intimal calcification is frequently seen in conditions related to atheroschlerosis [@Nakamura2009]. Medial calcification, also known as Monckeberg's arteriosclerosis, increases in prevalence in populations with increased age, diabetes mellitus, chronic kidney disease, chronic inflammation, and genetic disorders. [@Micheletti2008]

2.1.1 Arterial Calcification due to Deficiency of CD73 (ACDC)

Nonsense mutations in the NT5E gene, coding for ecto-5'-nucleotidase (CD73), have been discovered to cause medial arterial calcification of the limbs, in otherwise health individuals. (St Hilaire et al. 2011)

2.1.2 Arterial Calcification due to Deficiency of CD73 (ACDC)

2.1.3 Medial Calcification

2.1.4 Intimal Calcification

2.2 Mechanisms Behind Medial Arterial Calcification

Whole Mechanism: (Nitschke and Rutsch 2012): Genetics in arterial calcification: lessons learned from rare diseases. General review and also includes PIT2 (Rutsch, Nitschke, and Terkeltaub 2011): Genetics in arterial calcification: pieces of a puzzle and cogs in a wheel.

2.2.1 Tissue Non-specific Alkaline Phosphatase (TNAP)

(Henthorn et al. 1992): Different missense mutations at the tissue-nonspecific alkaline phosphatase (TNAP) gene locus in autosomal recessive inherited forms of mild and severe hypophosphatasia (Hessle et al. 2002):

Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization (Hoemann, El-Gabalawy, and McKee 2009): In vitro osteogenesis assays: Influence of the primary cell source on alkaline phosphatase activity and mineralization (Hotton et al. 1999): Differential Expression and Activity of Tissue-nonspecific Alkaline Phosphatase (TNAP) in Rat Odontogenic Cells In Vivo Phosphate (Jono et al. 2000): Phosphate Regulation of Vascular Smooth Muscle Cell Calcification - in vitro assays

2.2.2 Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 (ENPP1)

2.2.3 Phosphoethanolamine/Phosphocholine Phosphatase (PHOSPHO 1) and Pyridoxal Phosphate Phosphatase (PHOSPHO2)

2.2.4 ATP-binding Cassette Sub-Family C Member 6 (ABCC6)

2.2.5 Sodium-dependent Phosphate Transporter 1 (PIT1)

2.2.6 Progressive Ankylosis Protein Homolog (ANKH)

METHODS:

2.3 Other Monogenetic Diseases Related to Mechanism

2.3.1 Generalized Arterial Calcification of Infancy (GACI)

2.3.2 Pseudoxanthoma elasticum (PXE)

2.3.3 Potential Therapeutical Interventions

2.4 Animal Models

2.5 Quantitative Systems Modeling in Biology

2.5.1 Modeling Techniques

[@Gutenkunst2007]: Systems biology models are universally “sloppy”, meaning they that they contain many insensitive parameters and their behaviors are determined by relatively few number of stiff parameters.

2.5.2 Enzyme Kinetics

3 Experimental Design and Methods

3.1 Overview

3.2 General Protocols

3.3 Specific Aim 1

3.3.1 Strategy and Rationale

Proposed mechanism shown in Figure 1.

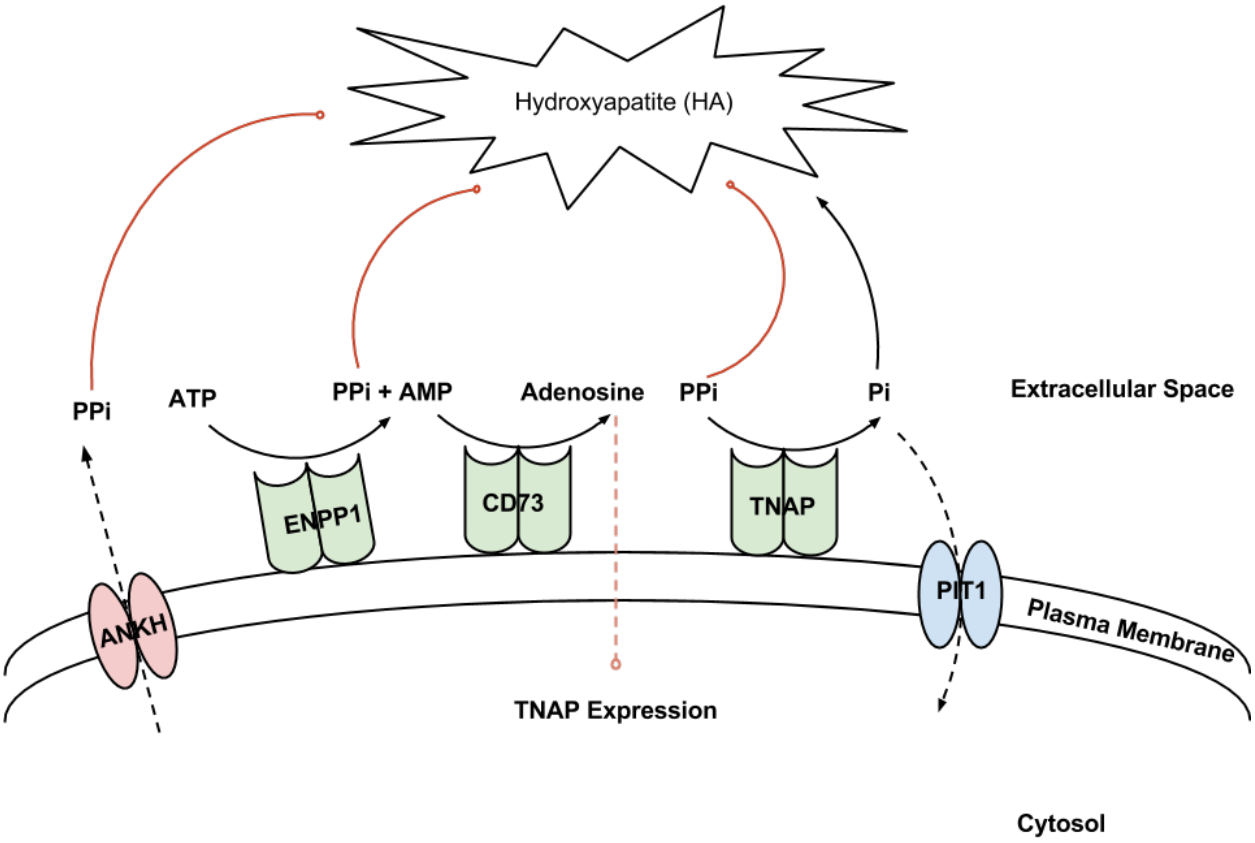

Figure 1: Proposed biological pathway involved in arterial calcification caused by ACDC, GACI, and PXE.

3.3.2 Experimental Plan

3.3.2.1 *In Vitro* Culture of VSMCs

3.3.2.2 Establish Methods for Measuring Key Metabolites

3.3.2.3 Quantify Enzyme Expression

3.3.2.4 Develop Antibodies for Highly Selective Blocking and Isolation of Pathways

3.3.3 Expected Results and Proposed Alternatives

3.4 Specific Aim 2

3.4.1 Strategy and Rationale

Equations 1 - **??**

$$\frac{d[\text{ATP}]}{dt} = -(\text{ENPP1 Activity}) - (\text{ATP Degradation}) + (\text{ATP Generation}) \tag{1}$$

$$\frac{d[PP_i]}{dt} = +(\text{ENPP1 Activity}) - (\text{TNAP Activity}) - (PP_i \text{ Degradation}) + (PP_i \text{ Generation}) \tag{2}$$

$$\frac{d[\text{AMP}]}{dt} = +(\text{ENPP1 Activity}) - (\text{CD73 Activity}) - (\text{AMP Degradation}) + (\text{AMP Generation}) \tag{3}$$

$$\frac{d[\text{Adenosine}]}{dt} = +(\text{CD73 Activity}) - (\text{Adenosine Degradation}) + (\text{Adenosine Generation}) \tag{4}$$

$$\frac{d[P_i]}{dt} = +(\text{CD73 Activity}) - (\text{TNAP Activity}) - (P_i \text{ Degradation}) + (P_i \text{ Generation}) \tag{5}$$

Filling out with Michaelis-Menten Kinetics Equations 6 - **??**

$$\frac{d[\text{ATP}]}{dt} = -\frac{V_{max\text{-ENPP1}}[\text{ATP}]}{K_{m\text{-ENPP1}} + [\text{ATP}]} - \alpha_{\text{ATP}}[\text{ATP}] + \gamma_{\text{ATP}}[\text{ATP}] \tag{6}$$

$$\frac{d[PP_i]}{dt} = +\frac{V_{max\text{-ENPP1}}[\text{ATP}]}{K_{m\text{-ENPP1}} + [\text{ATP}]} - \frac{V_{max\text{-TNAP}}[PP_i]}{K_{m\text{-TNAP}} + [PP_i]} - \alpha_{PP_i}[PP_i] + \gamma_{PP_i}[PP_i] \tag{7}$$

$$\frac{d[\text{AMP}]}{dt} = +\frac{V_{max\text{-ENPP1}}[\text{ATP}]}{K_{m\text{-ENPP1}} + [\text{ATP}]} - \frac{V_{max\text{-CD73}}[\text{AMP}]}{K_{m\text{-CD73}} + [\text{AMP}]} - \alpha_{\text{AMP}}[\text{AMP}] + \gamma_{\text{AMP}}[\text{AMP}] \tag{8}$$

$$\frac{d[\text{Adenosine}]}{dt} = +\frac{V_{max\text{-CD73}}[\text{AMP}]}{K_{m\text{-CD73}} + [\text{AMP}]} - \alpha_{\text{Adenosine}} + \gamma_{\text{Adenosine}} \tag{9}$$

$$\frac{d[P_i]}{dt} = +\frac{V_{max\text{-CD73}}[\text{AMP}]}{K_{m\text{-CD73}} + [\text{AMP}]} - \frac{V_{max\text{-TNAP}}[PP_i]}{K_{m\text{-TNAP}} + [PP_i]} - \alpha_{P_i}[P_i] + \gamma_{P_i}[P_i] \tag{10}$$

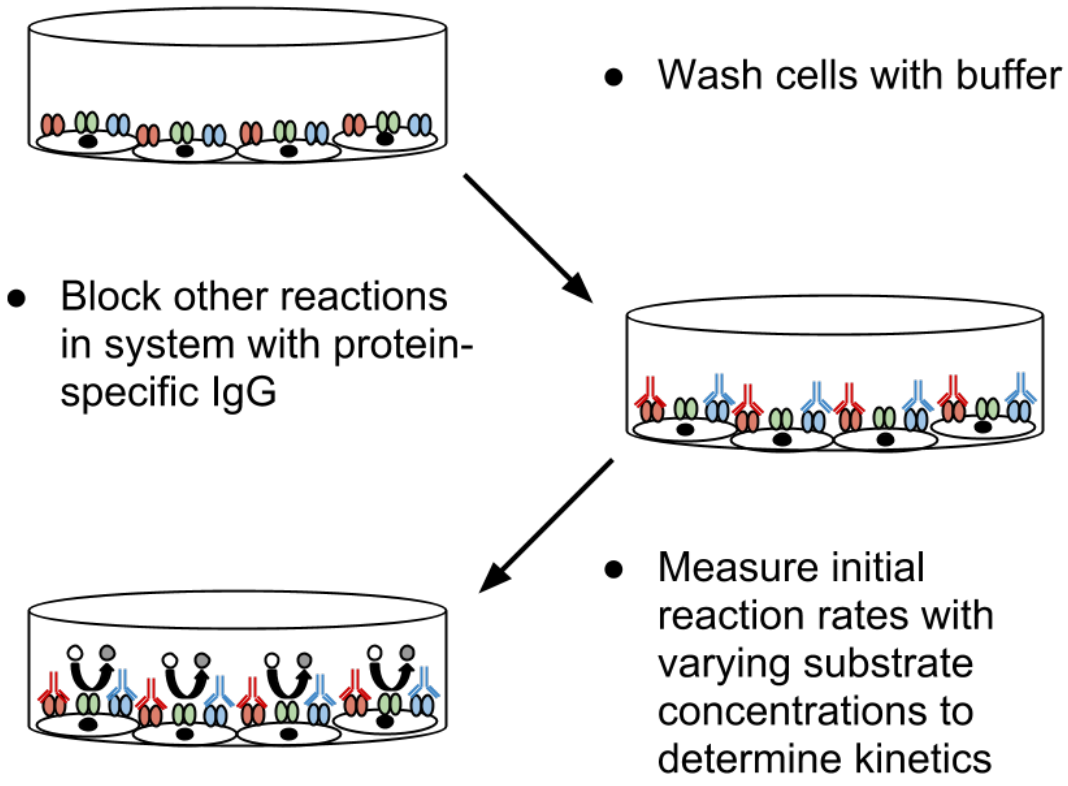

Figure 2: Generalized process for measuring enzyme kinetics.

3.4.2 Experimental Plan

3.4.2.1 Characterize and Quantify Enzyme Kinetics Through Initial Rate Reactions

3.4.2.2 Establish Confidence in Model Through Uncertainty Analysis and Cross Validation with Experiments

- Latin Hypercube

3.4.3 Expected Results and Proposed Alternatives

- Try reversible hill equation instead of Michaelis-Menten
- Consider alternative mechanisms

3.5 Specific Aim 3

3.5.1 Strategy and Rationale

3.5.2 Experimental Plan

3.5.2.1 Identification of Therapeutic Targets *In Silico*

- Sensitivity Analysis
- Flux Balance Analysis

3.5.2.2 Measurement of Therapeutic Efficacy on CD73 -/- Murine Model

3.5.3 Expected Results and Proposed Alternatives

4 Summary and Future Directions

5 Exam Question

William Gahl, the NIH sleuth who has identified a number of rare diseases, recently found that deletion of the gene NT5E led to calcification in leg arteries, to arterial insufficiency, and to inability to walk. The gene codes for an ecto-5'-nucleotidase, CD73. Write a proposal to support research to define the mechanisms by which the genetic abnormality causes the disease, and to find out how one can treat the disease. (St.Hilaire C, Ziegler SG, Markello TC, Brusco A, Groden C, Gill F, Carlson-Donohoe H, Lederman RJ, Chen MY, Yang D, Siegenthaler MP, Arduino C, Mancini C, Freudenthal B, Stanescu HC, Zdebik AA, Chaganti RK, Nussbaum RL, Kleta R, Gahl WA, and Boehm M. NT5E mutations and arterial calcifications. New Eng J Med 364: 432-442, 2011.)

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