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CD73 and the differences between mice and humans

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Abstract

As vascular disease is complex and the various manifestations are influenced by differences in vascular bed architecture, exposure to shear and mechanical forces, cell types involved, and inflammatory responses, *in vivo* models are necessary to recapitulate the complex physiology and dynamic cellular interactions during pathogenesis. Murine knockout models are commonly used tools for investigators to study the role of a specific gene or pathway in multifaceted disease traits. While valuable, these models are not perfect and this is particularly true in regard to CD73, the extracellular enzyme that generates adenosine from AMP. At baseline, CD73-deficient mice do not present with an overt phenotype, while CD73-deficient humans present with the complex phenotype of vascular calcification, arteriomegaly and tortuosity, and calcification in small joints. Below we highlight differences between the mouse and human systems and discuss the potential to leverage findings in mice to inform us on the human conditions.

Keywords

CD73; adenosine signaling; rare disease; vascular calcification; tortuosity; murine models; cardiovascular disease; VASCULAR DISEASE

The genetic origins and study of the molecular mechanisms driving rare diseases provide a unique window through which we can more fully understand human pathobiology. Rare genetic diseases may uncover novel mechanisms that regulate homeostasis or drive detrimental pathology, and disease discovery can highlight true gaps in our knowledge and, perhaps more importantly, the limitations of the model systems we use. This latter situation is precisely what we and our colleagues encountered with our discovery of the genetic cause of a disease termed Arterial Calcification due to Deficiency of CD73 (ACDC or CALJA, OMIM 211800) that presents with extensive vascular calcification, vessel tortuosity, and joint-capsule calcification.¹

Vascular calcification is the most striking phenotype in ACDC patients. Ectopic calcification in the vasculature is associated with a wide range of cardiovascular disease states and

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DISCLOSURES

None.

strongly correlates with increased cardiovascular risk. It was first identified in advanced atherosclerotic plaques and assumed to be an unregulated and non-biological consequence of degeneration, until more sophisticated imaging techniques identified small calcifications in early-stage plaques, and intriguingly, in the medial layer of otherwise healthy adults.²⁻⁴ It is now appreciated that both atherosclerotic (intimal) and vessel wall (medial) vascular calcification are active biological processes that alter vascular homeostasis and exacerbate disease pathologies, and vascular calcification is used as a risk-predicting biomarker.⁵⁻⁹ Currently there is no clinical treatment that halts or reverses pathologic calcification.

The genetic cause of ACDC phenotypes are mutations in the gene *ecto-5-prime-nucleotidase* (*NT5E*, NM_002526).¹ *NT5E* encodes for the protein CD73 (EC 3.1.3.5), an extracellular enzyme responsible for the conversion of AMP into adenosine and inorganic phosphate. The discovery of the genetic cause of ACDC was the first to link extracellular CD73 activity and its downstream adenosine receptor signaling to vascular calcification and tortuosity pathogenesis in humans. At the time of this discovery, several CD73 knockout mouse lines were available, yet these models do not present with a baseline phenotype that resembles what is seen in CD73-deficient patients.¹⁰⁻¹² Interestingly, much of the current research on CD73 is in the inflammation and cancer fields, and several clinical trials involving anti-CD73 monoclonal antibodies are currently being conducted for the treatment of solid tumors. As immunotherapy and pharmacotherapy focused on CD73-mediated signaling is gaining popularity, it is important to understand the implications of systemic effects of CD73 blockade. In this review, we aim to cover the role of CD73 in various organ systems to highlight how studies from the inflammation and cancer fields may inform our cardiovascular studies, and vice versa.

Adenosine Signaling

ATP is released from cells under conditions of stress (e.g. flow and mechanical stress, inflammation, hypoxia) or cellular death and is rapidly broken down. CD39 takes ATP to ADP and ADP to AMP in a two-step reaction yielding two inorganic phosphate molecules (Pi); ENPP1 breaks down ATP to AMP and pyrophosphate (PPi); and CD73 converts AMP to adenosine and Pi.¹³⁻¹⁶ Adenosine is referred to as a “retaliatory metabolite” and functions as a signaling molecule that allows cells to adapt to the initial ATP-releasing stress, however, overabundance of adenosine can induce damage; thus, concentrations of extracellular nucleosides must be tightly regulated.^{17, 18} Further fine-tuning of extracellular nucleoside concentration is regulated via equilibrative nucleoside transporters (ENTs) and pannexin transporters.^{19, 20} Adenosine signals by binding one of four G-protein coupled adenosine receptors (ARs) which are expressed on a wide range of cells and upregulated under various conditions; the density and combination of ARs on a particular cell will dictate the downstream pathways activated as their individual affinity to adenosine varies.²¹ The A2a and A2b ARs are classified as G_s-type receptors while A1 and A3 ARs are classified as G_{i/o} receptors, however it is now understood that AR signaling can be mediated through a variety of pathways.²²

ACDC Phenotype

Periarticular calcification

Case reports as far back as 1914 describe patients with ACDC-like phenotypes in the lower-extremity vessels.^{23–25} Secondary phenotypes associated with these cases of vascular calcification are early-onset arthritis and non-rheumatologic and intermittent joint pains caused by calcifications of the metacarpal phalangeal and interphalangeal joint capsules.^{1, 25, 26} Joints in the hands and feet of ACDC patients typically have bulky periarticular calcifications with mild joint space narrowing that is worse proximally and without intra-articular calcifications. The joint pain in ACDC patients is dynamic, waxing and waning throughout adulthood.^{1, 26} One patient was observed to have cyclical changes in mineralization, with exacerbations in pain occurring every 2–3 months. While still under observation this patient was enrolled in a clinical trial testing whether the bisphosphonate etidronate is effective in attenuating the progression of lower extremity arterial calcification and improving vascular blood flow (NCT01585402); the intermittent cyclic pain continued, and interestingly some bulky calcifications resolved while new bulky calcification developed. It is unclear whether these dynamic changes are characteristic of the normal disease pathogenesis, thus other patients with ACDC should be monitored to characterize disease progression.²⁶

Vascular calcification

The most extraordinary phenotype observed in ACDC patients is the vascular calcification. It is localized in the peripheral arteries and is exacerbated in vessels near joints of the lower extremities (e.g. iliac, popliteal, anterior tibial).^{1, 27} Since the initial discovery of ACDC in 2011, additional patients have been identified and the phenotype has expanded to include calcifications in the brachial artery near the elbow (see Table 1).^{28, 29} Symptoms include generalized lower extremity pain resulting from vascular incompetence and calculated ankle-brachial indices of less than 0.8. The calcification is non-atherosclerotic, found in the medial layer of affected vessels, and is accompanied by medial dysplasia consisting of an abundance of disorganized vascular smooth muscle cells, an infiltration of immune cells in the vessel wall, and significant deposition of extracellular matrix proteins. Together, this remodeling leads to vessel occlusion and vascular incompetence. More common forms of medial calcification are often non-obstructive, as with Mönckeberg arterial sclerosis,³⁰ but the severe medial calcification in ACDC extends into the vessel lumen and patients develop collateral vasculature as a compensatory mechanism.²⁷

As mentioned above, there is an ongoing clinical trial that is attempting to ameliorate ACDC calcification with the bisphosphonate, etidronate (NCT01585402). The use of a bisphosphonate to treat ACDC patients emerged from its ability to halt and in some cases reverse arterial calcifications in young patients suffering from a related inheritable disease, Generalized Arterial Calcification of Infancy (GACI, OMIM # 208000).^{31–34} Like, ACDC, GACI is an autosomal recessive disorder that presents with extensive medial layer vascular calcification, however, the vascular calcification in GACI patients is global and patients often die in infancy or early childhood due to cardiac failure. ENPP1 is an extracellular enzyme that is upstream of CD73 and catalyzes the breakdown of ATP into AMP and

pyrophosphate. Mutations in *ENPP1* are the cause of GACI.³⁴ When given bisphosphonates, 65% of GACI patients enrolled survived past infancy, compared to a survival rate 31% when not treated with bisphosphonates.^{35–37} Etidronate decreased the formation of calcifications in an *in vitro* ACDC calcification model and reduced existing calcifications in an *in vivo* ACDC-iPSC teratoma model; with the completion of NCT01585402 we will see whether what was modeled in this hybrid *in vivo* system can be mirrored in humans.³⁸

Arteriomegaly and Tortuosity

Arteriomegaly and tortuosity are characteristic of ACDC and phenocopies what is seen in aneurysmal remodeling.^{1, 39} In addition to medial thickening and calcification in the peripheral arteries, ACDC patient vessels exhibit duplication, fragmentation, and loss of curvature of elastin fibers.²⁷ This is most notable along the internal elastic lamina where small calcifications seem to nucleate at these break points. In ACDC we do not know what is happening first: does the calcification induce breakage of the internal elastic lamina, or does breakdown of this structure allow for nucleation of minerals? Looking to other connective tissue disorders provides strong evidence that matrix modulation and calcific deposition are intimately connected. Microcalcifications have been observed along elastin breakpoints in a dilated aorta from a Marfan Syndrome patient,⁴⁰ in mid-sized arteries and veins of patients with pseudoxanthoma elasticum (PXE),⁴¹ and large and mid-sized vessels in GACI.⁴²

CD73 in Mice and Humans

How does a lack of CD73 and its downstream adenosine signaling promote calcification and tortuosity? Are these phenotypes co-dependent or progress independently? To address these questions, *in vivo* disease modeling would be ideal. CD73-deficient mice were first created in 2004, and these early studies explored diverse organ systems and were found to have alterations in renal homeostasis¹⁰, decreased time to thromboocclusion¹¹, and pathologic responses to hypoxia (see Table 1).¹² Several conditional knockout models have since been created to explore the role of cell-specific CD73 function in heart failure, tumor growth and metastasis, and intestinal colonization and infection.^{43–45} Regarding calcification pathophysiology, CD73-deficient mice have pro-mineralization serum based on elevated Pi/PPi ratios but phenotypically present only with calcifications in the cartilaginous tissues of the sternocostal junction, juxta-articular tissues in the lower extremities and the knee, and the Achilles tendon.⁴⁶ However, CD73-deficient mice do not phenotypically mirror CD73-deficient humans in terms of medial vessel wall calcification, arteriomegaly, and vessel tortuosity. This not only complicates the ability to model this disease *in vivo*, but also emphasizes the point that mice are not little humans.

Possibly contributing to this particular human-murine disparity is the kinetics of adenosine. In human blood, the half-life of adenosine is less than 15 seconds, while in mice the half-life is approximately 2 minutes.⁴⁷ This difference may be due to the lack of the gene *CECR1*, which encodes for adenosine deaminase 2 (ADA2), in the murine genome. ADA2 serves a crucial role in human vascular homeostasis as those with ADA2-deficiency develop widespread vasculitis that presents with systemic necrotizing inflammation of blood vessels as well as aneurysms and stenosis of abdominal arteries.^{48, 49} This pathobiology is driven by

defects in endothelial cell integrity which induces in early-onset stroke and intracranial hemorrhage.⁴⁹ Ensembl gene tree ENSGT00530000063775 shows that *NT5E*, the gene encoding of CD73 protein, is present in 170 species, while Ensembl gene tree ENSGT00390000012118 shows *CECR1/ADA2* is present in 140 species. Noticeably absent from *CECR1/ADA2* gene tree are several model organism species, including the mouse, rat, Chinese hamster, Guinea pig, and rabbit. The lack of *CECR1/ADA2* may be contributing to the longer half-life of adenosine in mice compared to human counterparts. To date, there is no murine model that overexpresses human ADA2 to test this hypothesis, which renders studying adenosine formation, processing, and signaling in mice for the purposes of understanding human pathobiology, more challenging. *CECR1/ADA2* mutations are hypothesized to result in excessive amounts of adenosine, and mutations that induce ACDC inactivate enzymatic activity and result in too little adenosine. Comparing these two diseases highlights the importance of having the perfect balance of adenosine signaling in different vascular beds.

To work around these differences between mice and humans with CD73 deficiency such that we could identify the mechanisms contributing to pathologic calcification, we created induced pluripotent stem cells (iPSCs) from control and ACDC patient fibroblasts.³⁸ Tissue nonspecific alkaline phosphatase (TNAP) processes extracellular pyrophosphate (PPi) into phosphate (Pi) and is a key protein necessary for bone formation. We found that in CD73-deficient cells, TNAP compensates for the lack of CD73 by converting small amounts of AMP to adenosine, however, the adenosine produced was not to the same level as that seen in cells from control patients. As TNAP is upregulated, it preferentially converts PPi, an endogenous inhibitor of mineralization, to Pi, creating a pro-mineralization state. These effects were rescued by using inhibitors for PI3 kinase or MEK1/2, by treating cells with rapamycin, or by administration of an agonist specific for the A2bAR.³⁸

While the *in vitro* and *in vivo* disease modeling helps to identify the biological mechanisms contributing to the calcific phenotype, it does not inform us on the mechanisms driving tortuosity and changes in extracellular matrix. Instead of mice, should we look to species lower on the evolutionary tree? The use of zebrafish and drosophila (*Danio rerio* and *Drosophila melanogaster*, respectively) are heavily leveraged by the Undiagnosed Disease Network for the speed in which knockout or transgenic lines can be made.^{50, 51} In addition to sharing over 70% of their genome with humans, the vasculature of the zebrafish is easily visualized for real-time imaging.^{52, 53} Zebrafish were used to model ADA2-deficiency and show that reducing expression of *CECR1* paralogues induced intracranial bleeding, thus mirroring the defects in vessel development or integrity seen in the human patients.⁴⁸ Zebrafish can also develop vascular calcifications and *enpp1* mutants develop calcification in soft tissues of the skin, cartilage, heart, intracranial space and notochord sheet.⁵⁴

Much of what we know about CD73-mediated adenosine signaling is generated from murine models, yet from the discovery of ACDC we should appreciate that these models are imperfect. These CD73-deficient models employed different methods of mutation generation and background strains (see Table 1), and studies have utilized mice of different age, diet, and sex (see Table 2), yet all CD73-deficient models lack a vascular phenotype despite all having an absence of CD73 enzymatic activity. While the blood may not mimic the

microenvironment of the vessel wall, it is tempting to speculate that differences in the kinetics of adenosine in the blood and tissue microenvironment contribute to the differences between CD73 deficiency in mice and humans. Is it possible that overexpressing ADA2 in the CD73-knockout mouse would humanize it such that the phenotypes will mirror those seen in ACDC patients? Would a CD73-deficient zebrafish mirror ACDC better than the mouse because ADA2 is conserved in its genome? Until such models are created, knowledge gained from the CD73-deficient murine studies done to date may help to inform the vascular phenotype of ACDC patients. Below, we highlight a few arms of research.

CD73 in Inflammation

CD73-mediated adenosine signaling is an inflammatory response modulator. The role of CD73 in T cell proliferation and function was first described in mice in 1989 and has since been shown to drive T cell differentiation and is reduced in inherited and acquired immunodeficiencies.^{55–58} Adenosine generated by CD39 and CD73 on regulatory T cells (Tregs) is immunosuppressive.⁵⁹ This is corroborated by evidence that the immunosuppressors dexamethasone and methotrexate work via increasing the expression of CD73 activity and promoting the release of adenine nucleotides, respectively.^{60, 61} Indeed the mechanism for methotrexate efficacy in the treatment of rheumatoid arthritis is by increasing levels of adenosine; though not rheumatoid-like, the absence of local CD73-mediated adenosine signaling is hypothesized to be the cause of the periarticular calcification seen in the joint capsules of ACDC patients.^{1, 62}

Due to early-onset joint pain there is a push to classify CD73 deficiency within the spectrum of rheumatologic disease.⁶³ Supporting this, there is evidence of low CD73 expression on synovial lymphocytes in more severe forms of juvenile idiopathic arthritis, and the presence of CD73 is protective in an established murine model of rheumatoid arthritis, suggesting an interplay between calcification and inflammatory processes in this localized environment.^{64,65} However, the joint pain in ACDC is nonresponsive to anti-inflammatories and there is no evidence of auto-immune disease.^{1, 29, 39, 63} Further research indicates that defective adenosine signaling by A3 adenosine receptor ablation results in articular cartilage degeneration by aggrecanase and collagenase activity of chondrocytes.⁶⁶ Thus, CD73-mediated adenosine signaling may be protective against cartilage degeneration via the A3 receptor through matrix modulators rather than inflammatory mechanisms; this idea of extracellular matrix modulation also links to the role of CD73-deficiency in breakdown of the elastin lamina in the vessel wall. There is little data whether joint involvement in ACDC is immune-mediated or primarily through mineralization abnormalities, however, we cannot ignore the possibility of an immune-mediated mechanism due to the role CD73 has in immunomodulation in murine models. Interestingly, adenosine signaling is also antinociceptive, which introduces the question: is the joint-capsule phenotype of ACDC patients immune driven or does the lack of local adenosine in these locations increase pain sensitivity independent of an immune response.^{67, 68}

A significant amount of research on CD73 is in the cancer field. The immunosuppressive effects of CD73-generated adenosine in murine models suggest a favorable milieu for tumor growth, thus blocking CD73 activity is currently being explored as a therapeutic target.⁵⁹

Higher CD73 activity is associated with poorer cancer prognosis,⁶⁹ and the extracellular fluid of solid tumors was found to have immunosuppressive levels of adenosine that mediated its effects via the activation of the A2aARs on T cells.^{70–72} CD73-deficient mice are resistant to carcinogenesis and exhibit a decrease in tumor invasion and progression.^{73–75} In a melanoma model, CD73-deficient mice exhibited a decrease in tumor growth when tumor cells themselves were also CD73 deficient, however, these results were not recapitulated when CD73 was specifically deleted in host endothelial and hematopoietic cells using the Tie2Cre⁺/CD73^{flox/flox} mouse.^{43, 76} Currently, there are several human clinical trials using anti-CD73 monoclonal antibodies as primary or combination treatments for cancer (Clinical Trials NCT02503774, NCT03381274, NCT02503774, NCT03616886, NCT03611556, NCT03334617). Among the known ACDC population, one patient had a calcified oligodendrioma, but it is unknown if these patients generally have decreased risk of neoplastic disease.¹

CD73-mediated inflammatory mechanisms have also been implicated in murine models of heart failure. Humans with heart failure show an increase in inflammation and cytokine production. Given what is known about T cell modulation by CD73, global CD73^{KO} and CD4Cre⁺/CD73^{flox/flox} mice (T cell specific) underwent transverse aortic constriction to model heart failure. Although neither genetic model had a comparative increase in heart weight compared to wild-type mice, both of these knockouts exhibited worse ejection fraction as well as an increase end diastolic volume and end systolic volume. Functionally, these global and T cell specific CD73 knockout murine models had higher levels of inflammatory cytokines and fibrosis.⁴⁵ This suggests that the anti-inflammatory mechanisms of CD73-generated adenosine, specifically on T cells, may be protective in heart failure. No human with CD73-deficiency is known to have heart failure, however, it will be interesting to monitor for this in these patients to determine if progression and disease burden is worse.

In light of the differences between CD73-deficiency in the vasculature of mice and humans, it will be interesting to see whether the murine immunomodulating effects of CD73 blockade on cancer will be reproducible in humans. Similarly, as calcified vessels exhibit an infiltration of immune cells, perhaps inflammation is also contributing to the calcific or tortuous phenotype seen in ACDC joints and blood vessels.

CD73 and Vascular Remodeling

Alterations in extracellular matrix homeostasis are a hallmark of aneurysmal disease, and clues regarding the mechanisms driving aneurysm formation have been gleaned from familial rare diseases such as arterial tortuosity syndrome, Marfan Syndrome and Loeys-Dietz syndrome.⁷⁷ Common to these aneurysmal diseases is the upregulation of TGF- β signaling.^{78–80} As ACDC tortuosity phenocopies aspects of aneurysmal disease, understanding the pathogenesis of tortuosity in CD73-deficient vessels may provide new insights on a role for CD73 and adenosine signaling in aneurysm formation, and possibly a relationship between TGF- β and CD73/AR signaling pathways. Supporting this, adenosine signaling has been implicated in regulating matrix protein crosslinking via lysyl oxidase (LOX) activity through cAMP-mediated adenosine signaling on vascular smooth muscle cells.⁸¹ The vessels of patients with inactivating mutations in *LOX* exhibit fragmented and

disorganized elastic fibers, as LOX is an enzyme responsible for matrix crosslinking of elastin and collagen fibers, predisposing patients to thoracic aneurysms.^{82, 83} The histopathology found in these LOX-deficient vessels is similar to the peripheral arteries of ACDC patients.²⁷ In light of these similar disease phenotypes our lab is conducting studies on the role of CD73-deficiency in altering the expression of matrix remodeling enzymes and determining whether CD73-mediated adenosine signaling intersects with the TGF- β signaling pathway.

The calcification seen in ACDC patients is non-atherosclerotic and imaging analysis shows that their coronaries and aorta are normal for their age group.¹ However, murine models present a complicated view of the function of CD73 in atherosclerosis. Apolipoprotein E (ApoE) knockout and CD73/ApoE double knockout (dKO) mice were fed a normal chow diet for 26 weeks and at this time CD73/ApoE dKOs showed increased atherosclerotic plaque formation compared to ApoE knockout mice. This was most prominent in areas of turbulent flow (e.g. aortic valvular cusp and aortic bifurcation).⁸⁴ Plaque size was larger in the CD73/ApoE dKOs, but composition of collagen content, extracellular matrix proteins, hyaluronic acid, and lipid content were not different between these strains, suggesting no overt signs of plaque instability. Plasma from the CD73/ApoE dKO mice exhibited higher levels of MCP-1, triglycerides, and endothelin-1. Plaques in the ApoE knockout showed that macrophages and other cells of the mesenchymal lineage upregulated CD73, suggesting that CD73 on these cells helps to attenuate the immune response and reduces atherosclerotic plaque progression; the precise cause of increased number of lesions and lesion size was not defined, but speculated to be due to endothelin-1-induced migration of vascular smooth muscle cells.⁸⁴

Another study fed male ApoE and CD73/ApoE dKOs a western diet starting at four weeks of age and continuing for 16 weeks. Opposite the findings with normal chow, CD73/ApoE dKOs fed a western diet exhibited less plasma triglycerides, total cholesterol, LDL, and smaller plaque size.⁸⁵ *In vitro* studies with human aortic smooth muscle cells showed that knocking down CD73 expression in the presence of fatty acid reduced migration, proliferation, and transformation into foam-like cells.⁸⁵ Obviously, the dietary differences contributed to the drastic disparities in atherogenesis in the models, but why at baseline is CD73-deficiency atheroprone while under a Western diet CD73-deficiency is anti-atherogenic? Yang et al speculate these mechanistic differences are due to CD73 and adenosine sensitivity to lipids, as previous literature suggests that high-fat diets decrease CD73 expression of adipocytes in muscle,⁸⁶ while high-fat diets increase CD73 expression of hepatocytes.⁸⁷ One study looking at aortic valvular disease compared wildtype and CD73-deficient mice fed normal chow or high fat diet for 15 weeks. The authors discovered that CD73-deficient mice on both diets develop aortic valve thickening from melanocyte accumulation that resulted in increased peak flow, which is consistent with early aortic stenosis similar to that induced by high-fat diets.⁸⁸ Similar to the findings of Yang et al., an A2aAR/ApoE double knockout decreased atherosclerotic lesion size of the aorta and aortic valve, suggesting that a lack of CD73-mediated adenosine signaling is an important regulator in the formation of atherosclerosis in the presence of lipids.⁸⁹ Complimentary explanations for these different findings may lie in age, sex, and specific strains of the mice (see Tables 1 & 2).

ACDC patients show no overt evidence of atherosclerosis, and they do not have hyperlipidemia or elevated cholesterol levels; from the murine studies of Buchheiser et al., one would hypothesize that ACDC patients would be more prone to atherosclerotic calcification, however that does not appear to be the case. To add complexity, serum CD73 was found to be elevated in human patients with severe peripheral artery disease, and it was also elevated in the vaso vasorum of developing plaques, however it was absent in mature occlusive PAD plaques.⁹⁰ Thus, in PAD, is the upregulation of CD73 a cause or consequence? Experimental models and tracking disease progression alongside CD73 levels in the serum are needed to determine if downregulation of CD73 contributes to PAD pathogenesis.

CD73 and thrombus formation

Platelet function and recruitment heavily rely on internal reserves of ATP and ADP that are released into the extracellular compartment after initial platelet activation; ADP is a platelet agonist while adenosine is a known inhibitor of platelet function.^{91, 92} It is then reasonable to assume that CD73 activity may impact platelet function by adenosine generation and thus, initial thrombus formation. This could have profound implications on other vascular pathology such as acute myocardial infarction or peripheral thrombus formation. In two murine studies, time to thromboocclusion after low doses of oxidative stress was significantly decreased, although minimally, in carotid arteries of CD73 knockout mice.^{11, 93, 94} Pharmacologic inhibition of CD73 via α - β -methylene-ADP (APCP) negated the anti-thrombotic phenotype of overexpressing CD39 (CD39^{OE}), an enzyme that converts ATP to AMP in a two-step removal of the phosphate groups, but genetic ablation of CD73 had no effect. The authors hypothesize that this discrepancy is due to APCP's unknown off-target effects and ability to inhibit ENPP1, which is upstream of CD39, though they speculate that the latter seems less likely due to low endothelial and monocyte expression. In relation to humans, Netsch et al. found that mesenchymal stem cells inhibited platelet function from CD73-generated adenosine.^{93, 94} Comparing this to ACDC, one patient underwent an embolectomy of the popliteal artery,³⁸ and studies are currently being conducted to determine whether ACDC patients have premature thromboocclusive coagulation phenotypes similar to the murine model.

Discussion and Gaps in Knowledge

The differences seen between the phenotypes of CD73-deficient mice and humans highlight the limitations of using the murine model systems for the study of human disease (see Figure 1). The creation of an adequate model is crucial as dissecting the mechanisms driving ACDC phenotypes may provide powerful insights for vascular calcification, matrix dysregulation, aneurysmal disease, arthritis, and pain modulation. *In vitro* studies should be conducted with medial layer cells, as this is the primary location of ACDC pathology; however, in light of the role of CD73 and adenosine signaling on modulating the extracellular matrix and inflammatory cell differentiation and signaling, it is important to develop co-culture or 3D organoid models to study the interplay between different cells and matrix components. *In vivo* studies to generate double knockout mouse models or to induce calcification by other means should also be pursued as perhaps a "second hit" may induce an ACDC-like

phenotype in CD73-deficient mice. Lastly, maybe by taking a step down the evolutionary tree we could identify a more useful model system.

As CD73 becomes an important target in cancer therapy, studying the disease mechanisms and effects of patients with inherited CD73 deficiency can better inform us of what patients receiving anti-CD73 monoclonal antibodies, and thus partially acquiring CD73 deficiency, may experience. Long term monitoring of consequences of receiving these antibodies is crucial as it may increase risk of vessel matrix dysregulation, and joint calcifications and pain, heart failure, and ectopic medial calcification, with particular attention being given to those with co-morbidities (e.g. diabetes, chronic kidney disease, osteoporosis, hypertension) who are more prone to this type of calcification. This new and interesting population undergoing experimental therapies could themselves be another model system.

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ABBREVIATIONS

A1AR	A1 Adenosine Receptor
A2aAR	A2a Adenosine Receptor
A2bAR	A2b Adenosine Receptor
A3AR	A3 Adenosine Receptor
ACDC	Arterial Calcification due to Deficiency of CD73
ADA2	Adenosine Deaminase 2
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
APCP	α - β -methylene-ADP
ApoE	Apolipoprotein E
ARs	Adenosine Receptors
ATP	Adenosine Triphosphate
CALJA	Calcification of Joints and Arteries
CD39	Cluster of Differentiation 39
CD73	Cluster of Differentiation 73

CECR1	Cat Eye Syndrome Chromosome Region, Candidate 1
dKO	Double Knockout
ENPP1	Ectonucleotide Pyrophosphatase/Phosphodiesterase 1
GACI	Generalized Arterial Calcification of Infancy
iPSC	Induced Pluripotent Stem Cells
KO	Knockout
LDL	Low-Density Lipoproteins
LOX	Lysyl Oxidase
NT5E	Ecto-5-Prime-Nucleotidase
Pi	Inorganic Phosphate
PPI	Pyrophosphate
PXE	Pseudoxanthoma Elasticum
TGFβ	Transforming Growth Factor beta
TNAP	Tissue Non-specific Alkaline Phosphatase

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HIGHLIGHTS

- CD73 deficiency in humans leads to the development of medial calcification and matrix remodeling of the lower-extremity arteries and joint calcifications, yet CD73 deficient murine models do not recapitulate the human vascular phenotype
- In spite of these drastic differences between mice and humans several clinical trials are currently testing anti-CD73 antibodies for cancer therapy.
- This review highlights the known differences between the mouse and human systems and discusses the potential to leverage findings in mice to inform us on the human conditions.

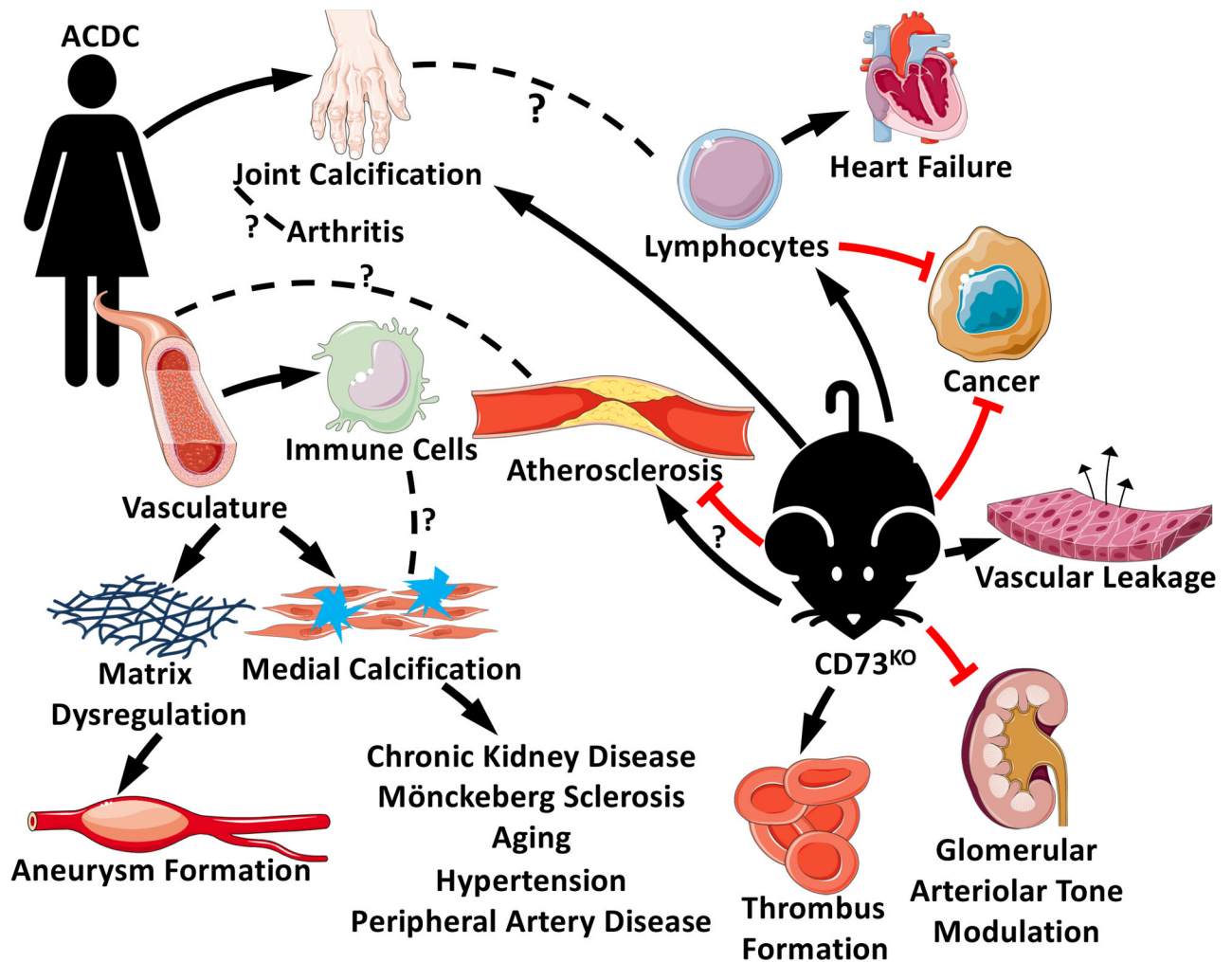


Figure 1. Summary of the Phenotypic Differences between CD73-deficient Mice and Humans.

At the 11 o'clock position, we represent a patient with ACDC and her associated phenotypes. Symptoms such as joint pain are due to extensive calcifications surrounding joint capsules, perhaps presenting an opportunity to study CD73 in distinct forms of arthritis. Vascular pathology is the predominant phenotype in ACDC with immune cell infiltration, elastic lamina degradation, and medial calcification, all resulting in symptoms and pathologies seen in more common vascular diseases. These phenotypes are in contrast with the murine CD73-deficient models represented by the image at the 4 o'clock position. Unlike their human counterpart, CD73-deficient models do not present with the same vascular pathology. Instead, studies have shown both a decrease and increase in atherosclerosis, and a decrease in time to thromboocclusion. These mice are less able to modulate glomerular arteriole tone and exhibit more vascular leakage in response to hypoxia. Perhaps the most thoroughly studied, cancer progression is decreased in most CD73-deficient murine studies in part by lymphocyte activity. Lymphocytes have also been shown to worsen heart failure in these models. Finally, these models can develop joint calcification, the only known phenotype that resembles human disease.

Table 1.
NT5E Mutations in Humans and Mice

A total of twelve patients from the reviewed literature have disease-causing mutations in *NT5E/CD73* resulting in ACDC. The patient of family 6 harbors four mutations including c.1086A>G p.T376A, however, his brother is homozygous for this same mutation yet is asymptomatic, thus this mutation unlikely to be causative. CD73-deficient murine models are also represented above. Models utilizing cell-specific knockdown of CD73 crossed cre-drivers to the CD73^{fllox/fllox} model.

Human Mutations		
Family	Mutation	Consequence
Family 1 (5 patients) ¹	c.662C>A (exon 3)	p.S221X; nonsense
Family 2 (3 patients) ¹	c.1073G>A (exon 5)	p.C358Y; missense
Family 3 (1 patient) ¹	c.662C>A (exon 3) c.1609dupA (exon 9)	p.S221X; nonsense p.V537fsX7; stop
Family 4 (1 patient) ⁶³	c.3G>C (exon 1) c.141C>G (exon 1) c.373G>C (exon 2)	p.M11 p.D47E p.E125X
Family 5 (1 patient) ²⁸	c.751+2T>C (intron 3)	Skips exon 3
Family 6 (1 patient) ²⁹	c.3G>C (exon 1) c.1096T>C (exon 5) c.1086A>G* c.488C>T	p.M11, start codon substitution p.M379T p.T376A silent
Murine Models		
Background	Mutation Location	Genetic Modification
C57BL/6J ¹⁰	Exons 2 and 3	Neo-Cassette
C57BL/6 ¹²	Exon 3	Neo-cassette
NMRI ¹¹	Exon 2	CD73 ^{fllox/fllox} (constitutive)
C57BL/6 ⁴³	Exon 2	Tie2Cre ⁺ /CD73 ^{fllox/fllox} Endothelial cell specific
C57BL/6 ⁴⁴	Exon 2	VillinCre ⁺ /CD73 ^{fllox/fllox} Intestinal epithelium cell specific
C57BL/6 ⁴⁵	Exon 2	CD4Cre ⁺ /CD73 ^{fllox/fllox} T-cell specific

Table 2.
Summary of CD73-Deficient Murine Studies

CD73-deficient murine studies highlighted within this review identify important scientific findings along with differences in model systems, age, sex, and diet. In studies with male and female rodents, no sex effects were observed.

Findings	Age (weeks)	Sex	Diet
C57BL/6J Neo-cassette (Exons 2 and 3)			
Decreased ability to alter glomerular arteriolar tone ¹⁰	8–16	M/F	normal
Juxta-articular joint-capsule mineralization, elevated serum Pi/PPi ratio ⁴⁶	44–64	M/F	high phosphorus, low magnesium
NMRI CD73^{flox/flox} (Exon 2)			
Decreased time to thromboocclusion (<i>Cre; full body</i>) ¹¹	8–12	not defined	normal
Increased atherogenesis (<i>Cre; full body</i>) ⁸⁴	24	not defined	normal
Increased peak aortic valve flow (<i>Cre; full body</i>) ⁸⁸	9+15 diet	not defined	high fat
Low CD73 expression on tumor melanoma cells inhibits tumor growth (<i>Cre; full body</i>) ⁷⁶	4–8	M	normal
CD73 on endothelial host cells does not modulate tumor growth or metastasis of B16-F10 melanoma cells (<i>Tie2-Cre; endothelial cell specific knockout</i>) ⁴³	14	M	normal
T cell specific loss of CD73 exhibits worse cardiac ejection fraction and an increase in end diastolic and end systolic volume (<i>CD4-Cre; T cell specific knockout</i>) ⁴⁵	8–12	F	normal
Increased nontyphoidal <i>Salmonella</i> colonization and virulence as well as reduced severity of colitis (<i>Villin-Cre; intestinal epithelial cell specific knockout</i>) ⁴⁴	not defined	not defined	normal
C57BL/6 Neo-cassette (Exon 3)			
Fulminant vascular leakage in response to hypoxia ¹²	52	M/F	normal
CD73 is protective in collagen-induced arthritis ⁶⁵	10–12	M	normal
Inhibits tumor progression ⁷⁴	not defined	M/F	normal
Resistance to carcinogenesis ⁷⁵	13–25	M	normal
Decrease in atherosclerosis ⁸⁵	4+16 diet	M	western
Decrease time to thromboocclusion; CD73 ablation does not decrease anti-thrombotic phenotype of CD39 overexpression ⁹³	8–12	M	normal