



Developing a Novel Pharmacotherapy to Target the Inflammatory Contribution to Cardiovascular Disease

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Statement of Originality

I certify that the intellectual content of this thesis is the product of my work, and that the assistance received in preparing this thesis and sources has been acknowledged. This thesis has not been submitted for any other degree or purpose. Examination of the Student Thesis is in accordance with university policies and procedures and the provision of examination results to the University. The research reported in this thesis was supported by the award of a Research Training Program scholarship to the PhD Candidate.

Elijah Samuel Genetzakis

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‘Για τη γιαγιά και τον παππούς μου, σας αγαπώ περισσότερο από όσο μπορώ ποτέ να πω’.

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Author attribution statement

Chapter One of this thesis contains work published as Genetzakis E, Gilchrist J, Kassiou M, Figtree GA. Development and clinical translation of P2X7 receptor antagonists: A potential therapeutic target in coronary artery disease? *Pharmacol Ther.* 2022;237:108228. I conceptualised and wrote this review and addressed all comments.

Chapter Three of this thesis contains data published as an abstract in Genetzakis E, Hansen T, Besnier M, Bubb K, Mcguire H, De Dreu M, et al. IL-1 β as a potential biomarker and therapeutic target in coronary artery disease. *Eur Heart J.* 2023;44(Supplement_2):ehad655-3137. I was responsible for data organisation, analysis, and data presentation. I drafted the abstract and handled revisions for presentation at the European Society of Cardiology.

Chapter Four of this thesis contains unpublished data submitted as an abstract to **E. Genetzakis**, YC. Chen, AMD. Watson, J. Noonan, O. Tang, G. Barsha, M. Kassiou, K. Peter, G. Figtree. Targeting the P2X7R with PKT100 stabilises atherosclerotic plaques in a mouse model of plaque instability. *Eur Heart J.* 2024. I designed the experiments in collaboration with Professor Karlheinz Peter and Dr. Yung Chih Chen's team, conducted blinded data analysis, and assembled the presented data. Dr. Yung Chih Chen conducted an initial blinded analysis in his laboratory and is an equal first author. I drafted the abstract with input from co-authors and will present the work at an upcoming conference.

Chapter Five of this thesis contains unpublished data submitted as an abstract to **Genetzakis E**, WE. Lee, G. Barsha, O. Tang, M. Besnier, M. Kassiou, GA. Figtree. Immunomodulation through P2X7R with PKT100 ameliorates diastolic dysfunction and fibrosis in obesity-induced cardiometabolic syndrome. *Eur Heart J.* 2024. I designed the experiments, conducted the data analysis, drafted the manuscript, and will present the work at an upcoming conference.

Chapter Six of this thesis contains unpublished data submitted as a provisional patent Adamantyl P2X7 receptor antagonists and their use in the treatment of cardiovascular diseases - Provisional patent, 2023904024, **28th July 2023**. I designed the cell-based experiments included in this patent, conducted dose-response curves on THP-1 and PBMC experiments, analysed and interpreted the data, and drafted the methods and results. My co-author Jayson Gilchrist contributed to the synthesis strategy for the abstract and Mr. Andre McKenzie was responsible for the cell dye uptake assay and analysis.

As the primary supervisor for the candidature upon which this thesis is based on, I can confirm that the authorship attribution statements above are correct.

Gemma Figtree
19/07/2024

Abstract

Over the past three decades, the implementation on secondary preventive strategies has reduced the global burden of cardiovascular disease (CVD). Despite this, recent years have seen a gradual increase in age-standardised risk and mortality from CVD, largely driven by the rising prevalence of metabolic disorders. Under these circumstances, there has been a refocusing on novel biological pathways to identify potential therapeutic strategies beyond traditional approaches. The Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) was the first to demonstrate that immunomodulation could effectively reduce the recurrence of major adverse cardiovascular events (MACE). This was demonstrated to be independent of lipid-lowering and confirmed the long-standing hypothesis that inflammation plays a significant role in the development and progression of atherosclerosis. Beyond its clinical implications, CANTOS provided evidence for a fundamental biological process in atherosclerosis, revealing the potential of the innate and adaptative immune systems which may play an early crucial role in atherosclerosis development. This was further corroborated by studies on SARS-CoV-2, where inflammation triggered by the infection was linked to accelerated atherosclerosis progression and increased risk of cardiovascular events. Thus, while the initial hypothesis which placed lipids at the centre of atherosclerosis development is not entirely invalid, it has become clear that the process is much more complex than previously understood with additional biological mechanisms yet to be discovered.

One potential candidate is the P2X purinoceptor 7 (P2X7R), a non-selective ATP-gated cation channel that mediates the influx of sodium and calcium ions and the efflux of potassium. The receptor is activated by high concentrations of extracellular adenosine-5'-triphosphate (ATP), which is released in response to cellular injury. This activation leads to the formation of a large, non-selective pore, resulting in sustained activation of the NOD-LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome complex. This complex plays a key role in the mechanisms required for the maturation and release of pro-inflammatory cytokines, such as interleukin-1 beta (IL-1 β). Moreover, the downstream consequence of P2X7R activation is the initiation pyroptosis via gasdermin-D (GSDMD). Compared to traditional immunomodulatory compounds like Canakinumab, which targets IL-1 β to neutralise its activity and may lead to immunosuppressive effects, targeting the P2X7R offers several advantages. Given the receptor is specifically activated in response to sterile damage-associated molecular patterns (DAMPs) such as ATP, inhibiting the P2X7R would help preserve immune mechanisms essential for host-defence. These properties make the P2X7R a promising therapeutic target in CVD, potentially paving the way for a novel therapeutic strategy.

Although P2X7R antagonists have not been evaluated in patients with CVD, several P2X7R antagonists have entered clinical trials in cohorts with rheumatoid arthritis (RA). Clinical trials in these cohorts initially showed promise but ultimately resulted in a neutral clinical outcome, leading to the discontinuation for the indication. In more recent studies, P2X7R antagonists have been investigated for neuroinflammatory conditions, where the P2X7R antagonist JNJ-54175446 exhibited improvements in mood in patients with major depressive disorder in

response to total sleep deprivation. Building on the moderate success of P2X7R antagonists in clinical cohorts, this thesis aimed to better translate findings using advanced patient-derived high-throughput pharmacological assays and in vivo mouse models that closely recapitulate human atherosclerotic plaque rupture. Furthermore, several series of P2X7R antagonists were developed, each characterised by variation in moieties and linker groups. The aim was to identify the optimal combination to improve the potency and pharmacokinetic properties of existing P2X7R antagonists. In the first results chapter of this thesis, cytometry by-time-of flight (CyTOF) was used to demonstrate enrichment of P2X7R expression on non-classical and classical monocytes, as well as CD1c⁺ myeloid dendritic cells (mDCs). These populations exhibited a strong association with coronary artery calcium score (CACs) and soft-plaque score (SPS) in patients with coronary artery disease (CAD). Ex vivo stimulation of monocytes enriched from PBMCs revealed enhanced P2X7R-dependent IL-1 β release following 2'(3')-O-(4-Benzoylbenzoyl) adenosine 5'-triphosphate (BzATP) stimulation in patients with CAD. The efficacy of the first-generation adamantane benzamide P2X7R antagonist PKT100 was then tested for its ability to reduce IL-1 β release in this cohort. The results showed that PKT100 reduced BzATP-induced IL-1 β release to near baseline concentrations. In patients with ST-elevated myocardial infarction (STEMI), P2X7R expression across CD1c⁺ mDCs and classical monocytes was significantly reduced and was negatively correlated with C-Reactive Protein (CRP) concentrations. Despite this, STEMI participants also demonstrated enhanced BzATP-induced IL-1 β release. Concomitant treatment with PKT100 reduced IL-1 β release to near baseline concentrations in this cohort, suggesting the potential significance of this pathway in atherosclerotic CVD.

Building on these results, the potential efficacy of PKT100 was next evaluated in a mouse model that mimics atherosclerotic plaque rupture. Six–nine-week-old male Apolipoprotein E knockout (ApoE^{-/-}) mice were fed a high-fat diet (HFD) for seven weeks. After this period, mice underwent tandem right carotid ligation to induce an unstable phenotype and were implanted with an osmotic minipump for continuous infusion of PKT100, a novel P2X7R antagonist over an additional 6-week period. The study demonstrated that PKT100 had significant effects on plaque stabilisation features, including reductions in neovascularisation and intraplaque haemorrhage, as well as substantial decreases in intraplaque lipids and increases in mature collagen fibres. These results were independent of a reduction in plasma lipids and further suggest a potential mechanism for plaque stabilisation in a tandem stenosis model that operates independently of lipid modification. These results further support the potential of a P2X7R antagonist as a therapeutic approach for the treatment of CAD. Expanding on these findings, the interplay between lipid and inflammatory pathways was evaluated given the focus on research in cohorts with heart failure with preserved ejection fraction (HFpEF). While those with CAD exhibit a substantial risk of HFpEF, which correlates with greater CAD severity, in general HFpEF is now considered the most common form of heart failure (HF), with repeated hospitalisation posing the greatest threat. In this study, C57BL/6J male mice that were 8-weeks old were fed a high-fat diet (HFD) for a total duration of 24 weeks. The results demonstrate that PKT100 treatment attenuated diastolic dysfunction and reduced interstitial fibrosis associated with HFpEF. These effects were accompanied by a significant reduction in the

expression of genes linked to inflammatory pathways, further supporting the role of P2X7R antagonism as a treatment in CVD.

In the final chapter, two novel series of P2X7R antagonists were developed and evaluated, with the aim to improve the potency and the pharmacokinetic properties of the first-generation antagonist. These compounds were also designed to provide new intellectual property and potential therapeutic applications for the treatment of CVD. The cyanoguanidine series, which utilised a novel linker originally developed by Abbott Pharmaceuticals, exhibited improved potency in the nanomolar range compared to earlier compounds. These compounds were demonstrated to be effective inhibitors of IL-1 β release across commercial cell lines and patient-derived monocytes from cohorts with CAD and STEMI. However, the development of these compounds was halted due to poor pharmacokinetic properties. In this instance, the half-life of these compounds in liver microsomes was limited to just a few minutes. The metabolic liability appeared to be inherent to the chemical structure, rather than being specific to a single entity, thus leading to the discontinuation of the series. Under this circumstance, there was a revision to the development strategy and a new series of compounds were developed based on the adamantane amide structure originally patented by AstraZeneca (AZ) in 2003. This approach involved the modification of the adamantane cage by replacing the hydrogen atoms with fluorine. These compounds demonstrated significant improvements in the pharmacokinetic properties, with a half-life surpassing that of the initial PKT100 compound in both human and rodents. Notably, the potency of this series was largely preserved, which supports the continued clinical development of these compounds as a potential treatment for CVD.

In summary, this thesis underscores the potential of P2X7R antagonists as a novel therapeutic strategy for CVD. Through the comprehensive use of patient-derived cells for pharmacological evaluation, in combination with risk stratification and preclinical evaluation, these findings provide a strong foundation for the development of P2X7R antagonists and offers a promising alternative to conventional anti-inflammatory treatments for CVD.

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Abbreviations

ACPAs	Anti-citrullinated protein antibodies
ApoE^{-/-}	Apolipoprotein E knockout
ASC	Apoptosis-associated speck-like protein containing CARD domain
ASCVD	Atherosclerotic cardiovascular disease
ATP	Adenosine-5'-triphosphate
AU	Agatston units
AZ	AstraZeneca
BPM	Beats per minute
BzATP	2'(3')-O-(4-Benzoylbenzoyl) adenosine 5'-triphosphate
CACs	Coronary artery calcium score
CAD	Coronary artery disease
CANTOS	The Canakinumab Anti-inflammatory Thrombosis Outcome Study
CD	Crohn's Disease
COLCOT	Colchicine Cardiovascular Outcome Trial
CRP	C-Reactive Protein
CTCA	CT coronary angiography
CVD	Cardiovascular Disease
CytoF	Cytometry by-time-of flight
DAMPs	Damage-associated molecular patterns
DCM	Dilated cardiomyopathy
DMSO	Dimethylsulfoxide
EC₅₀	Half-maximal effective concentration
EF	Ejection Fraction
ELISA	Enzyme-linked immunosorbent assays
FBS	Fetal bovine serum
GLS	Global longitudinal strain
GSDMD	Gasdermin-D
GSK	GlaxoSmithKline
HBSS	Hank's Balanced Salt Solution
HDL	High-density lipoprotein
HF	Heart failure
HFD	High-fat diet
HFpEF	Heart failure with preserved ejection fraction
HIBS	Heat inactivated bovine serum
IC₅₀	Half-maximal inhibitory concentration
ICAM-1	Intracellular adhesion molecule-1
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-18	Interleukin-18
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IFN-γ	Interferon-gamma

JUPITER	Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin
L-NAME	N ω -Nitro-L-arginine Methyl Ester
LDL	Low-density lipoprotein
LDL-R	LDL-receptor
LoDoCo	Low-dose Colchicine
LPS	Lipopolysaccharide
LV	Left ventricle
MACE	Major adverse cardiovascular events
mDCs	CD1c ⁺ myeloid dendritic cells
MI	Myocardial infarction
MTX	Methotrexate
NADPH	Nicotinamide adenine dinucleotide phosphate
NETs	Extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHMRC	National Health and Medical Research Council
NK cells	Natural killer
NLRP3	NOD- LRR- and pyrin domain-containing protein 3
NNT	Nicotinamide nucleotide transhydrogenase
P2X7R	P2X purinoceptor
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDCs	Plasmacytoid dendritic cells
PET	Positron emission tomography
PSAX	Parasternal short axis
PSLAX	Parasternal long axis
PSR	Picrosirius red
qPCR	Quantitative PCR
RA	Rheumatoid arthritis
SEM	Standard error mean
SGLT2	Sodium-glucose cotransporter 2 inhibitor
SPS	Soft-plaque score
STEMI	ST-elevated myocardial infarction
TGF-β	Transforming growth factor beta
TMD	Transmembrane domains
TNF-α	Tumour necrosis factor alpha
TREM2	Triggering receptor expressed on myeloid cells 2
TS	Tandem stenosis
VSMC	Vascular smooth muscle cells
WGA	Wheat-germ agglutinin
α-SMA	Alpha-smooth muscle actin

Chapter 1

Introduction

Traditional risks in cardiovascular disease

Over the last three decades, there has been a marked decline in the global burden of age-standardised cardiovascular disease (CVD) and mortality, particularly notable in some higher-income countries (**Figure 1A**).

(1) This positive trend is mainly owed to the effective implementation of population-based interventions and policies that address modifiable risks associated with atherosclerotic (AS)CVD, as well as improvements in the acute care of patients with myocardial infarction (MI). (1) Previous guidelines, informed by the findings from the Framingham Heart Study, implicated five ‘traditional’ modifiable risk factors, namely, hypertension, obesity, hypercholesterinemia, diabetes, and tobacco use as significant contributors to CVD risk. (2–4) Of the risk factors, advancements in preventative therapies, such as statins, anti-hypertensives, and glucose-lowering medications applied within a secondary preventative framework have contributed to the substantial reduction in age-standardised global burden. (1) Moreover, patient prioritisation for pharmacotherapeutic management of CVD has been improved with updated contemporary risk scores, with the inclusion of polygenic risk scoring. (5) However, despite these advancements, coronary artery disease (CAD) remains a major public health challenge and the single greatest driver of mortality in the world. Inequalities continue to persist around the availability of these treatments, where the global burden in lower-income countries, reflected by regions with low primary-based care, remains dormant. (1,6)

Nonetheless, these small victories in CVD are undermined by the increasing prevalence of age-standardised CVD in regions where the rates were previously declining. (1) The re-emergence of age-standardised risk is accounted for by the growing prevalence of metabolic disease driven by hyperglycemia, inadequate food nutrition, obesity, and a sedentary lifestyle. (1,7) Under these circumstances, current guidelines have been updated to include nutrition, physical activity, and sleep as disease management risks in preventing CVD. (4) Additionally, in cohorts with heart failure with preserved ejection fraction (HFpEF) - a group known to have a substantial metabolic risk and underlying atherosclerotic disease - disease-modifying agents such as sodium-glucose cotransporters 2 inhibitors, typically recommended for diabetes are being evaluated in heart failure (HF) outcomes. (8,9) However, when unadjusted for age, the incidences and mortality from CVD have continued to rise worldwide over the last 30 years, a feature attributed to the substantial growth and median age of the population. (1) Under these considerations, CVD has retained its notorious position as the lead cause of mortality and morbidity worldwide, with substantial room for therapeutic intervention. In our goal towards maximising healthy ageing and reducing sudden cardiac death, new approaches are required to work synergistically with more traditional approaches targeting the known risk factor.

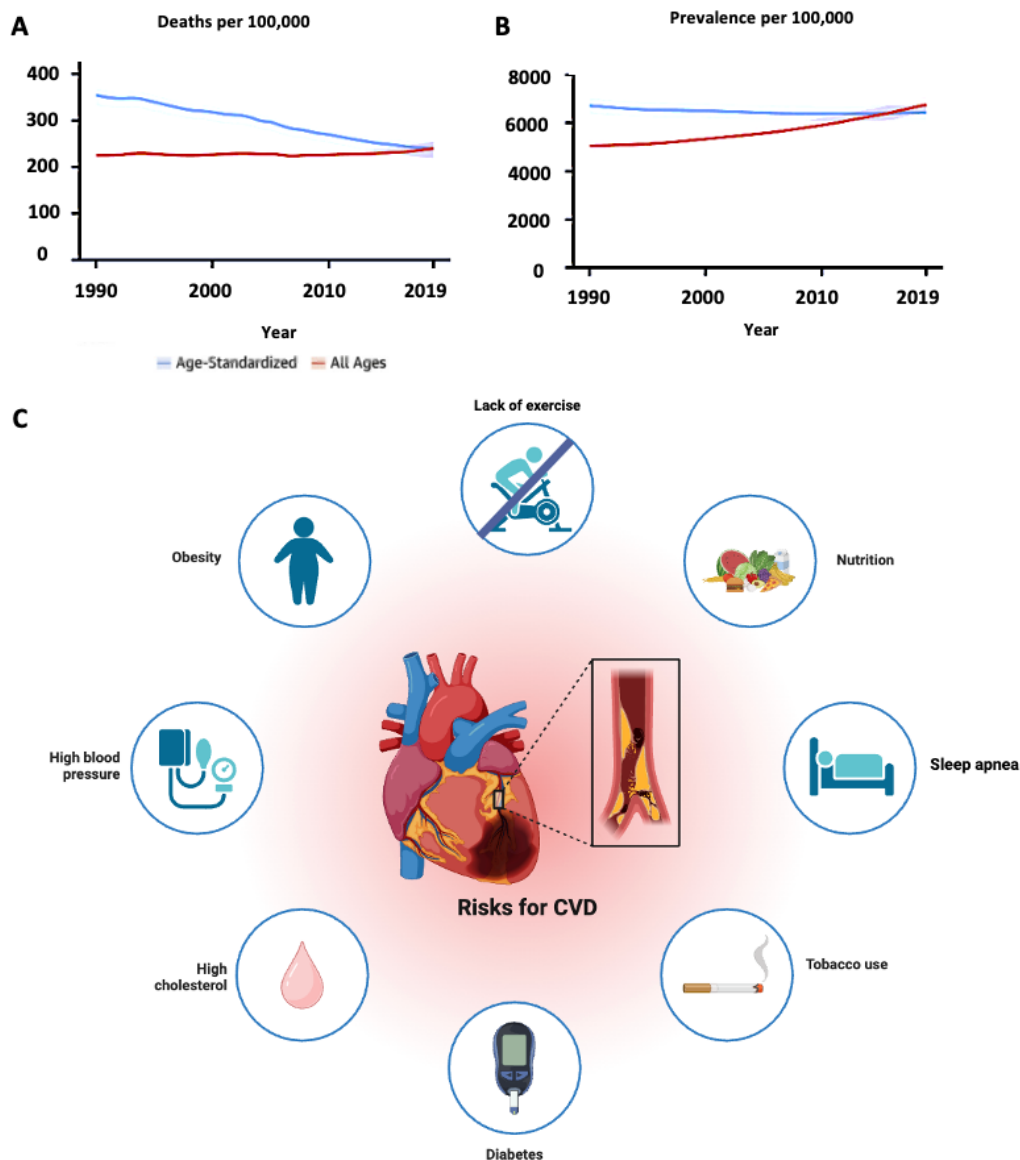


Figure 1: Cardiovascular disease is the leading cause of mortality and morbidity worldwide. A) The age standardised death rate per 100,000 from CVD was reduced from 1990 to 2019, whereas the non-standardised death rate gradually increased over this time. **B)** The prevalence of CVD per 100,000 after age-standarisation gradually reduces, while non-standardised cases show a sharp increase in prevalence, likely related to population growth and increased life expectancy. **C)** Eight key risk factors for CVD are now identified in updated guidelines: namely hypertension, obesity, sedentary lifestyle, nutrition, sleep, tobacco use, hyperglycemia, and hypercholesterolemia. **Created with BioRender.com.** Adapted from (1) Copyright Creative Commons Attribution License.

Inflammation as a critical therapeutic – beyond traditional risk factors

Recent data and analysis from the Global Cardiovascular Risk Consortium showed that key modifiable risk factors – hypercholesterolemia, hypertension, smoking, obesity, and hyperglycemia – account for 57.2% of 10-year incidences of CVD amongst women and 52.6% amongst men. (2) This suggests that up to 48% of the burden of

CVD might be attributed to unaccounted-for risk arising from unresolved biological mechanisms and is substantially less than previous large-scale studies such as the INTERHEART study, (10) which had suggested that up to 90% of the population attributable risk is explainable by nine modifiable risk factors. The pragmatic and clinical perspective of this unexplained CVD, particularly CAD, has been highlighted in a recent paper by Figtree et al., who demonstrated that 14 to 25% of first-time MI patients present culprit plaque development despite no traditional risk factors. (11) An international collaborative effort has been established to address the missing biology and unmet needs of these patients- specifically, new diagnostic tools for the detection of early atherosclerosis and treatments that target the plaque independently of risk factors. This is particularly true given that over 75% of patients have no symptoms before their emergency presentation. (3)

The JUPITER (Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin) determined that patients who showed both reductions in low-density lipoprotein (LDL) cholesterol and C-reactive protein (CRP), achieve better clinical CVD outcomes. (12) While CRP, commonly used as a surrogate marker of inflammation, was known to be heightened in patients with CVD, the clinical benefits associated with its reduction brought in a new era for targeting strategies and treatment approaches in ASCVD. However, it was not until the CANTOS Study (Canakinumab Anti-Inflammatory Thrombosis Outcome Study) that the field gained robust evidence demonstrating the clinical benefits of targeting inflammation in CVD. In this landmark trial, Canakinumab, an interleukin – (1β)-specific monoclonal antibody, reduced primary endpoints of nonfatal MI, nonfatal stroke, and mortality from CVD by 15%. (13) This benefit was independent of an effect of LDL that remained unaltered. (13) Additionally, key secondary endpoints, such as hospitalisation for unstable angina that led to urgent revascularisation, were reduced by 17%. (13) In a secondary analysis, CRP concentrations lower than 2 mg/L were associated with a greater reduction in major adverse cardiovascular events (MACE). (14) Patients who did not reach therapeutic reductions in CRP did not demonstrate a clinical benefit, suggesting an additional inflammatory risk independent of IL- 1β . Nonetheless, the CANTOS trial opened new avenues in the development of pharmacotherapeutics for the treatment of CVD and inspired a new decade of clinical outcome trials in patients with residual inflammatory risk.

C-reactive protein (CRP)

The relationship between CRP and future CVD event risk is now well established, as discussed above and is associated with unstable angina and MI. (15–17) Improvements in the reliability and sensitivity of CRP detection methods with high-sensitivity approaches have facilitated its role in clinical trials. (18) The detection of CRP in the clinical setting is associated with increased secretion of inflammatory cytokines, which indicate an underlying pathological process. (17) In response to an acute MI, elevated CRP concentrations in some studies correlate with infarct size, left ventricular ejection fraction, and circumferential strain. (19) However, CRP is suggested to have a limited casual role in the development of ASCVD, with the primary causal agent likely cytokines released upstream. CRP is synthesised by liver hepatocytes in response to the binding of interleukin-6 (IL-6) to the IL-6 receptor on liver cells. (20) Studies have shown that the transcription of *IL-6* can be induced

via interleukin-1 (IL-1) activation. (21) Particularly, IL-1 treatment of Caco-2 cells was demonstrated to increase *IL-6* gene expression via phosphatidylinositol 3-kinase-Akt-dependent pathways. (21) This relationship was confirmed in a secondary analysis of the CANTOS trial, where IL-1 β neutralisation resulted in a substantial reduction in IL-6 but not interleukin-18 (IL-18). (22) Despite the benefits of CRP as an enrichment biomarker or surrogate marker in early-phase human trials, its detection in serology is understood to be non-specific and not useful in detecting disease in patients with ASCVD. (23) Consequently, CRP is an indicator of inflammation, but beyond this, it is limited in detecting patients with CVD. Given the key role of inflammation in the residual risk of CAD, there is an unmet need for new markers that reflect the underlying disease activity and are associated with responses to treatments that benefit MACE.

IL-1 β

Decades of research have focused on the significance of IL-1 β on cardiovascular outcomes, ultimately resulting in the hypothesis behind the CANTOS trial. (13) As an upstream mediator of IL-6, IL-1 β neutralisation with Canakinumab demonstrated substantial benefits in reducing cardiovascular risk. (13) It is most well-known for its role in host defence in response to infection and injury. (24) The precursor form, a 31 kDa protein that is mostly inactive, is synthesised after nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation and translocation to the nucleus by pathogen-associated molecular patterns. (25) However, in sterile disease states like CVD and cancer, this mechanism can be attributed to non-pathogenic particulates, such as tumour necrosis factor alpha (TNF- α) and IL-1 β -mediated activation of NF- κ B. (26) Particularly, TNF- α acts through TNF receptor 1, where activation induces receptor-interacting protein 1 recruitment and ubiquitination, leading to NF- κ B activation. (27) The production of precursor IL-1 β , unlike other cytokines, is produced in two phases, where phase one is the 'priming' phase, and pro-IL-1 β accumulates in the cytoplasm. (28) For the production of the mature form, a second phase is required, primarily enabled by the enzymatic cleavage of caspase-1. (29) Caspase-1, activated by the NOD- LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome, (30) also cleaves gasdermin-D (GSDMD) between the C- and the N- terminus, where the N-terminus translocates to the cell membrane to form pores. (31) Of particular importance, these pores can facilitate rapid secretion of IL-1 β , while their involvement in cell destabilisation makes them integral in intracellular cytokine release. (31,32) Under these circumstances, IL-1 β is also released from apoptotic cells through passive secretion. (31)

The interleukin-1 (*IL-1*) family includes two distinct genes, *IL1A* and *IL1B*, which encode the proteins IL-1 α and IL-1 β (33) IL-1 α does not require caspase-1-dependent maturation and primarily remains membrane-bound (33), while the IL-1 β is promiscuous in its activity. However, unlike IL-6 and CRP, the detection of IL-1 β in plasma has been limited in large-scale epidemiological studies as its detection is unreliable. (34) Particularly, its detection is hindered by a short plasma half-life, with a maximum 2-hour duration proposed and a relatively low abundance compared to IL-6 and IL-18. (35) Moreover, circulating IL-1 β does not accurately reflect levels at a cellular or organ level where the release may be pertinent to the pathology. Despite this, IL-1 β remains an attractive

therapeutic target in CVD, given its long-standing association with innate and adaptative inflammation, supported by a substantial body of experimental and clinical evidence. While the CANTOS study proved preliminary evidence for the clinical benefit of IL-1 β reduction, there has yet to be an imminently translatable method of IL-1 β inhibition. Specifically, there are significant pragmatic issues related to the delivery of a monoclonal antibody for what is perceived as a chronic disease, together with safety concerns related to a small but significant increase in sepsis linked to the treatment. (13) This point will be discussed further in the thesis.

IL-6

The secretion of IL-6 is partly mediated through the release of IL-1 β , as discussed in the section above on CRP. (21) A secondary analysis on the CANTOS exemplified this relationship, where IL-6 concentrations below 1.65 ng/L were associated with a 36% reduction in MACE with Canakinumab. (36) Decrease in plasma IL-6 was also associated with a reduction in all-cause mortality, (36) suggesting an overall benefit of IL-6 reduction on health. Experimental evidence has shown that IL-6 acts as a secondary messenger cytokine, facilitating various functions related to both innate and adaptive immunity. (37) Activated immune populations such as monocytes, together with non-hemopoietic cells such as fibroblasts and smooth muscle cells, release IL-6. (38,39) In the context of CVD, IL-6 has garnered significant attention as a potential therapeutic and diagnostic biomarker in ASCVD. (40) It has been highlighted in human trials due to its strong association with the hepatic synthesis of CRP. (34) Apart from CRP, the elevation in the levels of IL-6 results in the synthesis of several other acute-phase proteins, such as fibrinogen and serum amyloid A, which have also been identified as potential markers of CVD risk. (41) Under these circumstances, IL-6, despite having a shorter half-life compared to CRP, is shown to be a better predictor of CVD risk and could be a causal factor in the progression of atherosclerosis. (42)

Indeed, in a prospective trial in healthy males, baseline levels of IL-6 were found to be heightened in those who went on to have a CVD event. (41) This study was the first in a healthy cohort to observe an increased risk for future MI predicted years before the event, suggesting a potential role of IL-6 in early atherosclerosis. (41) Moreover, evidence from genome-wide association studies confirmed that the missense mutation D358A [encoded by the single-nucleotide polymorphism (SNP) rs2228145] in the IL-6 receptor was associated with a reduced IL-6 mediated CRP production and vascular risk. (43) Based on this evidence, the RESCUE trial was established to evaluate the efficacy of Ziltivekimab, an IL-6 monoclonal antibody, in a cohort with chronic kidney disease and elevated CRP. (44) Although terminated early due to the COVID-19 pandemic, patients on 30 mg Ziltivekimab demonstrated a 92% reduction in CRP after 12 weeks. (44) Notably, this effect was twice as large as the reduction observed in the CANTOS trial and has inspired a large-scale trial evaluating the efficacy of Ziltivekimab, known as ZEUS. (45) Notably, the selection criteria for the study included participants with chronic kidney disease with a CRP concentration greater or equal to 2 mg/L and evidence of ASCVD defined by a history of MI coronary revascularisation procedure, greater than 50% stenosis in a major coronary artery or symptomatic peripheral artery disease. (45) The results of this outcome trial are scheduled to be released in 2025.

NLRP3 inflammasome

Efforts to translate epidemiological discoveries into clinical treatments have led investigators towards targets upstream of acute-phase proteins. The NLRP3 inflammasome has been the subject of substantial studies as its assembly was recognised to promote downstream caspase-1 activity and pro-inflammatory cytokine secretion. (46–49) The role of the NLRP3 inflammasome in cytokine maturation was recognised decades earlier due to defects in its structure that lead to a gain-of-function mutation. (50) Patients with this mutation present with excessive IL-1 β characterised by recurrent fevers, joint pain, and rash. (50) The assembly of the NLRP3 inflammasome is dependent on potassium and calcium efflux, primarily mediated by the P2X purinoceptor 7 (P2X7R) activation. (51,52) As the receptor is responsible for downstream IL-1 β and IL-18 maturation, and thus, IL-6 and CRP synthesis, its suppression from a pharmacological perspective is suggested to be more beneficial than single cytokine suppression. The NLRP3-specific inhibitor MCC950, shown to bind to the Walker B motif, prevents adenosine-5'-triphosphate (ATP) hydrolysis required for NLRP3 assembly. (53)

In a mouse model of diabetes-associated atherosclerosis, treatment with MCC950 was associated with a reduction in atherosclerotic lesions and markers of atherosclerotic plaque instability. (54) This was associated with a substantial reduction in the expression of proinflammatory cytokines TNF- α and IL-1 β , together with plasma secretion of IL-6. (54) The efficacy of MCC950 to protect against transaortic constriction-induced hypertrophy (TAC), when treatment 2 weeks after induction, was associated with better-preserved ejection fraction and less tissue-specific inflammation. (55) Interestingly, however, in a mouse model of atherosclerotic plaque instability, MCC950 did not improve markers of plaque destabilisation, nor did IL-1 β neutralisation alone. (56) In the case of MCC950 in this model, systemic inflammation was unchanged, which may explain the lack of efficacy, although IL-1 β neutralisation achieved significant reductions in systemic inflammation without affecting plaque stabilisation. (56) From an epidemiological perspective, a common SNP located in the intron-spanning region of the NLRP3 inflammasome was associated with a higher prevalence of CAD in participants under the age of 60 despite no differences in traditional risk factors. (57) This mutation was associated with higher systemic inflammation indicated by higher IL-18 and CRP in plasma. (57)

Colchicine, an ancient anti-inflammatory approved for use in patients with gout, was also shown to enact its anti-inflammatory properties through the NLRP3 inflammasome. (58,59) The Colchicine Cardiovascular Outcome Trial (COLCOT) evaluated the efficacy of Colchicine in a cohort with ASCVD. (60) Treatment with Colchicine was associated with a 65% reduction in CRP and reduced incidences of primary endpoints indicated by stroke and urgent hospitalisation for angina leading to revascularisation. (60) This was replicated in a subsequent study, Low-dose Colchicine (LoDoCo) in a stable atherosclerosis cohort, where the primary endpoint indicated by cardiovascular mortality, nonfatal MI, nonfatal stroke, and coronary revascularisation was reduced by 29.2%. (61) This implies a potential therapeutic advantage of upstream modulation in treating CVD.

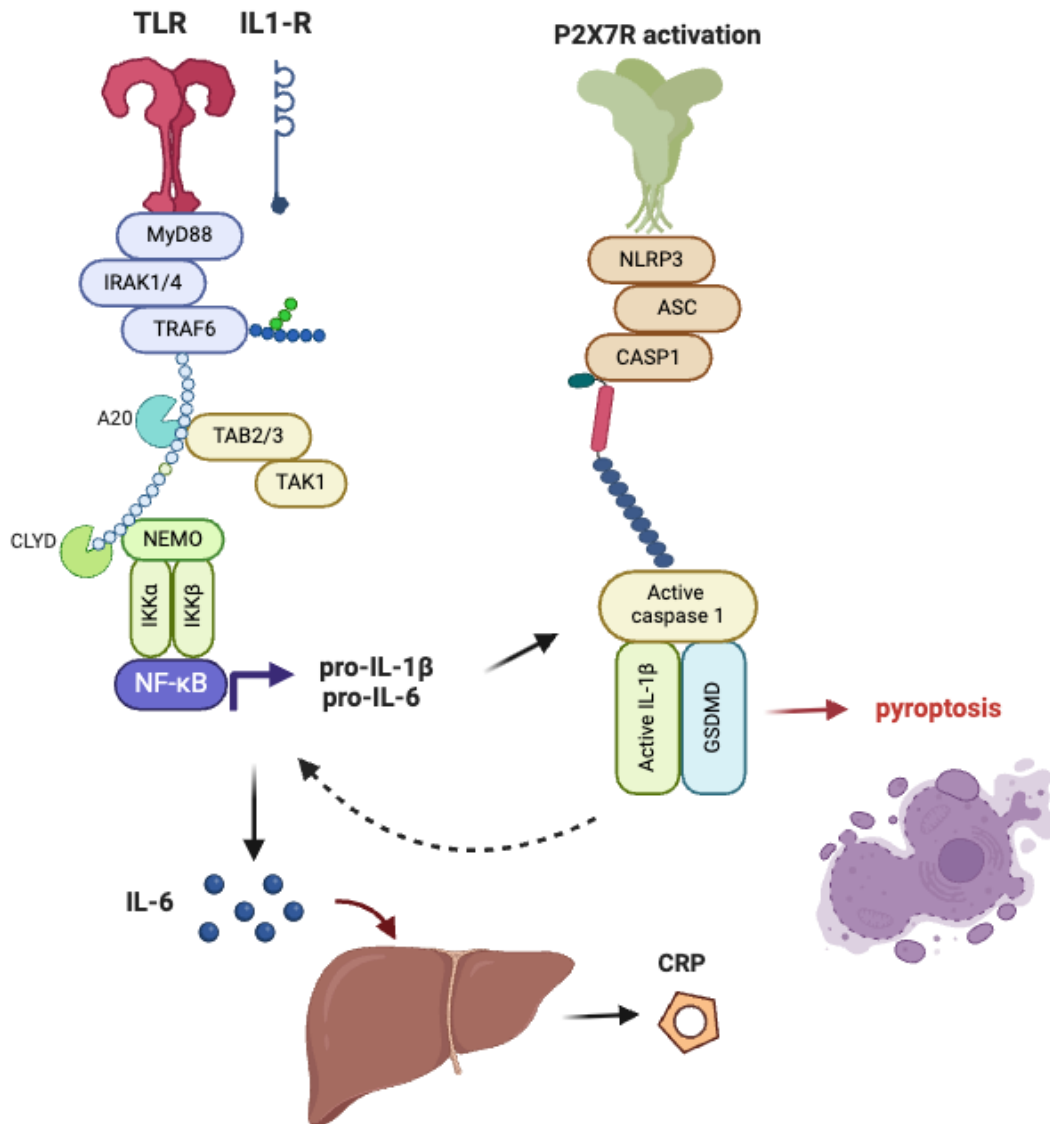


Figure 2. Identified markers of inflammation in ASCVD and their synthesis pathway. Inactive precursors IL-1β and IL-18 are synthesised after translocation of NF-κB to the nucleus to enhance transcription of the corresponding genes. Precursor cytokines IL-1β and IL-18 are stored in the cytoplasm and require additional processing for release. The P2X7R, activated under intensive inflammatory conditions, promotes the assembly of the NLRP3 inflammasome, responsible for the cleavage of caspase 1, an enzyme that transforms cytokines into their active forms. Increased caspase-1 activity results in the production of mature IL-1β and IL-18, but also GSDMD. The expression of GSDMD promotes pyroptosis of the cell but provides a mechanism of IL-1β and IL-18 secretion. Accumulation of IL-1β and IL-18 can affect NF-κB activity, producing IL-6 that does not require additional processing. IL-6 acts on the hepatocytes that make CRP, which has a relatively long half-life and is used as a general marker of inflammation. **Created with BioRender.com**

The current understanding of inflammation in cardiovascular disease pathogenesis

CAD

Atherosclerosis, as a primary cause of CAD, is defined as a systemic inflammatory disease that affects the arterial walls. (62) It is characterised by the deposition of Apolipoprotein B-containing lipoproteins in the tunica intima, a process mediated by the immune system. (63) For decades, atherosclerosis was postulated to be driven by oxidised LDL, where its migration into vascular walls leads to foam cell formation. (62) While parts of these mechanisms remain valid, our previous understanding of the disease was flawed and largely ignored the contribution of inflammation in the development of atherosclerosis. Arterial endothelial cells that act as a protectant between the vasculature and blood become activated in response to various signals, such as systemic inflammation and disruptions in blood flow. (64) Their activation is characterised by the expression of adhesion molecules, including vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 (ICAM-1) . (65) Additionally, the production of vasoactive substances such as endothelin-1, angiotensin 2, and nitric oxide modulate vasodilation and promote the adhesion of monocytes that participate in vascular inflammation. (66,67) Their migration to the subendothelial space and differentiation into macrophages regulate their phagocytic response, where clearance of LDL occurs through macrophage ingestion of LDL particles. (68) At first, this response is necessary for the adequate clearance of LDL, but later, when overwhelmed, it becomes pathogenic, contributing to the first phase of symptomatic atherosclerosis, referred to as the foam cell. (68) These mechanisms, particularly the transmigration of adherent monocytes, are directed by chemokines released from other immune cells. However, the use of single cell-based technologies has further stratified macrophages, with triggering receptor expressed on myeloid cells 2 (TREM2)^{hi} macrophages found to promote lipid uptake. (72) In a murine model of atherosclerosis, TREM2^{-/-} mice confirmed a role for this macrophage subset, where mice demonstrated lower plaque burden than their littermates. (72) Comparatively, the loss of TREM2^{hi} was also associated with increased necrotic core formation due to its role in efficient efferocytosis and foam cell survival. (72,73)

Efferocytosis describes a mechanism by which macrophages, with their phagocytic function, recognise and remove apoptotic cells based on their surface 'eat me' expression. (74) This process aims to resolve atherogenesis and overburdens macrophages, culminating in their death and necrotic core formation in atherosclerotic plaques. (75) Under these circumstances, the necrotic core is marked by acellularity and occupied by lipids, subjecting the plaque to a vulnerable phenotype. Increased necrotic core volume in clinical cardiology is associated with future cardiovascular event risk and myocardial ischemia. (76,77) The formation of necrotic cores involves a complex interplay between the synthesis of lipid species and inflammation. From the perspective of efferocytosis, TREM2^{hi} macrophages, otherwise known as 'foamy' macrophages, exhibit cholesterol transport and metabolism genes. (78,79) Comparatively, genes related to inflammation in these macrophages are downregulated. (78) A second subset of macrophages, characterised as 'non-foamy', exhibit upregulated expression of inflammation-related genes, such as those coding for IL-1 β and NLRP3. (78) This subset may represent newly recruited macrophages coming from dendritic or vascular smooth muscle cells

(VSMC) origins. (63,78) A third subset identified as TREM1^{hi}/PLIN2^{hi} macrophages exhibits a unique signature of both lipid and inflammation-related genes. (80) It is predicted that the TREM2^{hi} macrophages can transition to a TREM1^{hi}/PLIN2^{hi}, while the reverse is not true. (80) Their transition is associated with localisation to the necrotic core cleft and warrants further investigation into the contribution of this macrophage plaque destabilisation. (80)

Macrophages, as critical orchestrators of inflammation in atherosclerosis, have a vital role in the degradation of the extracellular matrix. (81) The synthesis of glycoproteins such as collagen by VSMC provides structural stability and mechanical strength to the extracellular matrix of the plaque. (81) Atherosclerotic lesions prone to rupture feature large collagen-deficient necrotic cores beneath a thin fibrous cap, marked by the accumulation of intraplaque lipids. (82) The contribution of other immune populations involved both in adaptative and innate inflammation in atherosclerosis is often overlooked, given the considerable involvement of macrophages in plaque development and progression. However, CD4⁺ and CD8⁺ T cells comprise more than 65% of the total immune cell populations in atherosclerotic plaques. (83) In ruptured atherosclerotic plaques, their transcriptional profile was associated with a gradual reduction in T cell function, indicated by the expression of markers known in T cell exhaustion, activation, and differentiation. (83) Concomitant expression of activated and exhausted populations in culprit lesions suggests sustained inflammation, where chronic exposure to autoantigens depletes these cells of their inflammatory capabilities. (84) While the exact consequence of this phenotypic switch is not well understood in human unstable plaques, mouse studies demonstrate that CD8⁺ T cells accelerate atherosclerotic plaque development. (85) Aged mice in these studies showed an increased proportion of CD8⁺ T cell exhaustion marked by thymocyte selection-associated HMG box protein and programmed death-1. This suggests the potential deleterious role of T cell exhaustion in atherogenesis through enhancement in inflammatory genes. (83,86)

The rupture of atherosclerotic lesions as a result of plaque destabilisation is a common cause of MI and stroke. (87) In the context of ST-elevated MI (STEMI), where complete occlusion of the coronary artery results in ischemia and necrosis to the myocardium, there is a crucial interplay between inflammation and appropriate myocardial repair. (88) Compared to neonates, adults have limited regenerative capacity due to the activation of the Hippo-YAP pathway known to regulate the development of organ growth and size. (89) This cell-intrinsic mechanism is essential to prevent adverse cardiac development. (90) After MI, the heart is challenged by the same paradigm, where cardiomyocyte loss due to ischemic injury is replaced by non-function collagen due to the limitations in cardiac regeneration. (91) Under these circumstances, effective immunomodulation of myocardial repair is essential to ensure sufficient heart function. (92) The release of damage-associated molecular patterns (DAMPs) from injured cells, such as ATP and heat shock proteins, act on toll-like receptor-4 to promote cytokine secretion. (93,94) The activation of the innate immune system starts with the rapid synthesis of neutrophils from the bone marrow and their infiltration into the myocardium. (95) As early responders, neutrophils are programmed to internalise cellular debris, produce reactive oxygen species,

degranulate, and form extracellular traps (NETs). (96) While these mechanisms are crucial in the fight against infections, NET formation was found to contribute to lesion development in atherosclerosis and was associated with heightened inflammation. (97) Mice deficient in PR3 and NE, which are critical components of NETosis demonstrate smaller atherosclerotic lesions and less inflammation compared to their counterparts. (98) Neutrophils were also found to be deleterious in atrial fibrillation post-MI through the release of oxidative proteins. (99,100) Macrophages were demonstrated to be protective against post-MI arrhythmias, although these populations have been previously characterised as detrimental in other studies. (100)

Post-MI, macrophages constitute the largest population of immune cells in the heart, with a dynamic transcriptional signature. (95) While in early inflammation, they demonstrate a proinflammatory signature (days 1-3), between days 5-7, they transition to an anti-inflammatory state characterised by expression of TREM2^{hi}. (95) Macrophage-specific TREM2^{hi/-} mice demonstrate heart function reductions associated with decreased left ventricular (LV) wall thickness after MI. (101) From the perspective of the myocardium, TREM2^{hi} macrophages were found to have a significant effect on myocardial remodelling through efferocytosis, (95) suggesting a deleterious effect of macrophage depletion strategies. Comparatively, in the initial inflammatory phase, excessive inflammation mediated by macrophages is proposed to prevent appropriate myocardial repair. (92) Under these circumstances, suppression of inflammation in this early phase is postulated to improve clinical outcomes. Although macrophages and neutrophils have been outlined as crucial contributors in the resolution phase post-MI, a substantial body of literature recognises the key role of cardiomyocytes in inflammation.

The contribution of ischemic damage, altering cardiomyocyte metabolism due to vascular occlusion, results in cardiomyocyte apoptosis. (102) Within border regions, these cardiomyocytes are acknowledged as at-risk populations that must adapt to the new demands of the heart. (101) Populations in remote areas, characterised as the region juxtaposed to the ischemic region, also undergo significant modifications to protect against HF. (103) While the cardiomyocytes release lower concentrations of cytokines, apoptosis after injury releases various inflammatory signalling molecules such as ATP, heat shock proteins, nuclear and mitochondrial DNA, and HMGB1. (104) These molecules can act downstream through several pathways, leading to the sustained release of inflammatory cytokines that impede adequate resolution post-MI. (88) The toll-like receptor-4 and the subsequent NF-κB pathway raise inflammation in the ischemic zone, leading to further cardiomyocyte apoptosis. (105) The VCUART3 evaluated this hypothesis in human trials with the IL-1R antagonist, Anakinra. (106) Reduction in inflammation determined by CRP was associated with better survival within 360 days, particularly related to an improvement in death or hospitalisation for HF. (106) These results were replicated in the COLCOT trial that evaluated the efficacy of low-dose Colchicine in a population with a recent event within 13.5 days. (60) Based on this evidence, timely inflammatory inhibition has emerged as a crucial management strategy post-MI. Moreover, the expression of anti-inflammatory immune phenotypes in later stages proves to be advantageous in wound repair, offering a new therapeutic opportunity for the management of inflammation post-MI.

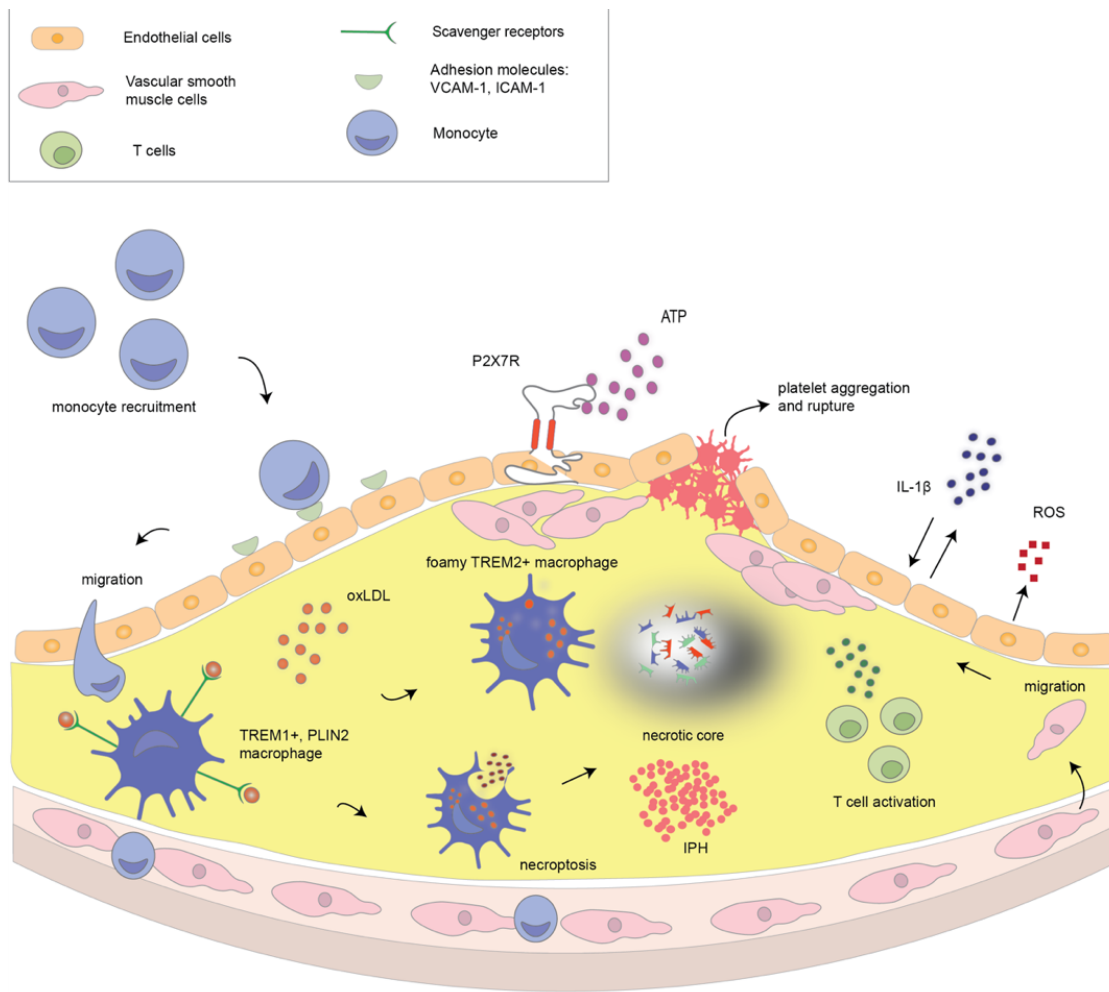


Figure 3. Inflammation plays a critical role in the progression and development of atherosclerosis. The expression of adhesion molecules on endothelial cells promotes the transmigration of monocytes to the subendothelial space and promotes their diversification to macrophages. Macrophages in the subendothelial space primarily function to clear oxidised LDL particles. Their death is associated with the ‘foamy’ cell appearance and the presentation of atherosclerotic plaque. Macrophages with an inflammatory phenotype, marked by IL-1 β expression, infiltrate the plaque to perform efferocytosis—removal of dead cells. However, this process can become overwhelmed, forming a necrotic core characterised by acellularity and a loss of extracellular matrix. T cells activated in plaque also have three major phenotypes. Particularly, exhausted T cells, which are largely inefficient in their functions, are associated with plaque destabilisation through enhanced inflammation. These processes lead to neovessels that form under the pressures of hypoxia, resulting in the deposition of intraplaque hemorrhage linked with unstable features in complex atherosclerotic plaque. The destabilisation of plaque marked by a thin fibrous cap, migration of VSMC to the border, and necrotic core expansion are associated with a rupture-prone phenotype. The lipid-rich necrotic core becomes exposed to the blood contents, where adhesion molecules lead to the attachment of thrombi, mainly constituting platelets. These plaques contribute to death from stroke or MI. Adapted from (182) **Created with Adobe Illustrator.**

Cardiometabolic syndromes

The implementation of secondary preventative strategies for the treatment of ASCVD has contributed to the significant decline in the occurrence of mortality from MI, together with nonfatal MI and stroke events. (4) Simultaneously, the rising prevalence of obesity, hypertension, and insulin resistance has increased incidences of HFpEF. (107) From a clinical perspective, the presentation of HFpEF, characterised by diastolic dysfunction, cardiac hypertrophy, and interstitial fibrosis, impairing LV relaxation, is correlated with the prevalence of metabolic syndrome. (108) Its presentation is proposed to be higher amongst women compared to men, where women tend to be older and have worse clinical outcomes. (109) Independently, CAD in women with HFpEF was associated with heightened susceptibility to primary endpoints measured by CVD mortality or hospitalisation due to HF. (109) Indeed, for some time, it has been difficult to disentangle the molecular mechanisms underlying the clinical presentation in HFpEF, primarily due to the complex heterogeneity of the phenotype. (102) It is now recognised that HFpEF is a clinical feature rather than a distinct disease state, where its presentation coexists with various comorbidities. (110) Amongst these, the cardiometabolic presentation, marked by obesity, insulin resistance, hypertension, and hypercholesterolemia, has emerged as the most prevalent form. (102) Previous human trials have aimed to target the myriad of clinical manifestations, particularly through the SGLT2, where Empagliflozin, a management strategy for type-2 diabetes, was shown to reduce the incidence of hospitalisation for HF. (9) While hospitalisation outcomes improved in those with a LV ejection fraction below 50%, treatment with Empagliflozin failed to demonstrate a mortality benefit across risk stratification. (9) Trials with Semaglutide, aimed to reduce weight through glucagon-like peptide 1, demonstrated improvements in HF-related symptoms compared to placebo. (111) This study was, however, underpowered to report on the efficacy of Semaglutide in CVD mortality; hospitalisation outcomes due to HF improved slightly. (111) While addressing insulin resistance and obesity for the management of HF associated with HFpEF are viable therapeutic options, it is evident that further therapeutic strategies are yet to be elucidated.

The contribution of metabolic stress is associated with the activation of the adaptative and innate immune systems in HFpEF pathophysiology. (102) In particular, the inflammatory marker CRP was shown to be reduced in patients treated with Semaglutide after a reduction in body weight in the STEP-HFpEF trial. (111) Patients with HFpEF were shown to have increased expression of T and B cells in cardiac tissue, where their expression was associated with increased transforming growth factor beta (TGF- β) expression. (112) CD3⁺ and CD4⁺ T cells, with a distinct role in antigen recognition, were found to be upregulated in the myocardium in an established two-hit model of HFpEF. (113) Mice deficient in T cells through T cell receptor alpha knockout demonstrated preserved diastolic function and reductions in cardiomyocyte hypertrophy despite treatment with a high-fat diet (HFD) and Nw-Nitro-L-arginine Methyl Ester (L-NAME), a nitric oxide inhibitor. (113) While other immune cells were not evaluated, there is clear evidence of the involvement of T cells in myocardial remodelling associated with an HFpEF phenotype. In previous studies, patients with HFpEF exhibited an increased proportion of PBMC-derived T helper 17 compared to regulatory T and CD4⁺ T helper cells. (114) This relationship was further heightened in PBMCs from patients with HFpEF and was correlated with N-terminal pro-brain natriuretic peptide

(114) This is suggestive of a mechanism of action of T cells in myocardial inflammation in the context of HF in the general sense.

Indeed, T cell receptor alpha knockout mice under TAC were used to explore these mechanisms. T cell receptor alpha knockout mice showed preserved diastolic heart function, improved survival, and reduced cardiomyocyte hypertrophy and fibrosis. (115) Notably, TGF- β , a marker of inflammation, was also reduced. (115) In age-related studies, CD4⁺ T cells in aged mice were demonstrated to be upregulated compared to young mice. (116) The expression of this population was associated with an upregulation of inflammation and diastolic dysfunction, whereas the ablation of the B cell population did not influence myocardial inflammation. (115) The expression of inflammatory cytokines is hypothesised to have a significant role in oxidative stress produced by coronary microvascular endothelial cells, limiting the bioavailability of nitric oxide to cardiomyocytes. (117) Reductions in the availability of nitric oxide are suggested to reduce the activity of protein kinase G in cardiomyocytes, contributing to their growth. (117)

A review of inflammation in metabolic-driven HFpEF syndromes would only be complete with a short discussion of the macrophage populations. Similarly to their involvement in CAD, pre- and post-MI, macrophages are assigned a distinct role in tissue efferocytosis and inflammatory resolution. (102) The myocardium contains a resident macrophage population that undergoes dynamic expansion in response to acute hemodynamic stress. (118) Macrophages, derived from bone marrow-produced monocytes, contribute to the expansion of myocardial macrophage populations in HFpEF and is associated with increases in oxidative stress and inflammation. (118) Depletion of macrophages in an established HFpEF model reduced systemic release of IL-1 β and normalised diastolic measures to near the baseline, indicative of the role of macrophages in obesity-induced HFpEF. (119) These pathways were previously explored in mice with fatty acid binding protein 4 knockout, which exhibit reduced secretion of cytokines such as, IL-1 β . (120) The reduction in these pathways in fatty acid binding protein 4 knockout mice was associated with improvements in diastolic parameters, despite the presence of elevated fasting glucose concentrations, equivalent to control mice. (119) Elevations in IL-1 β related to diastolic dysfunction were linked with heightened mitochondria superoxide production, (119) where the HFpEF heart demonstrates dysregulation in oxidative balance. As acute responders to microenvironment alterations, macrophages release cytokines such as interleukin-10 (IL-10) in a hypertensive-induced HFpEF murine model. (118) The release of IL-10 is strongly associated with age, where aged mice demonstrate higher concentrations of IL-10, with increased expansion of Mac3⁺ macrophages. (118) IL-10 release induced a fibrogenic phenotype, where myofibroblast differentiation and activation are associated with collagen deposition in cardiac tissue. (118)

The deposition of collagen in the interstitium is linked to myocardial stiffness in diastolic dysfunction. Monocytes from peripheral blood were increased in patients with HFpEF compared to those with hypertension. (121) This was linked with heightened plasma cytokine secretion, indicated by TNF- α , monocyte chemoattractant protein

1, and IL-6. (121) Interestingly, however, gene expression from monocytic populations was not associated with a distinct difference in inflammatory cytokines, while expression of anti-inflammatory, pro-fibrotic cytokines was upregulated in plasma and monocytes from HFpEF patients, such as IL-10. (121) The heightened expression of proinflammatory cytokines, attributed to the higher proportion of macrophages/monocytes, has been previously highlighted in a clinical study, where ‘unstable HFpEF’, characterised by acute decompensated HF, was associated with increased plasma concentrations of IL-6, TNF- α , and CRP compared to those with stable disease. (122) In this study, IL-6 concentration predicted rehospitalisation events due to HF after age adjustment. (122) Although the mechanisms of HFpEF remain elusive, the emergence of inflammation to the phenotype offers potential avenues for drug discovery. Particularly, strategies for the management of HFpEF remain scarce outside of the treatment of traditional risk factors. Thus, targeting inflammation offers a potentially novel mechanism in therapeutic development aimed at improving outcomes for patients with HF.

Challenges in the development of anti-inflammatories for cardiovascular disease

Despite decades of data implicating inflammation in atherosclerosis pathophysiology, it was not until recently that immunomodulatory therapies have been shown to benefit cardiovascular outcomes in human clinical studies. This next section will discuss a selection of these compounds, the outcomes of the trials, and their relevance to the inflammatory ‘hypothesis’ in CVD. The details of these trials are summarised in Table 1.

Canakinumab

From a cardioimmunology perspective, it would be almost inappropriate not to start with the story of Canakinumab. Canakinumab, an approved monoclonal antibody developed to neutralise the of IL-1 β is approved for cryopyrin-associated periodic syndrome and Muckle-Wells syndrome. (123,124) Both of these conditions are autosomal dominant and arise from mutations in the *NLRP3* gene – a multimeric complex and result in the excessive production of IL-1 β . (46) The CANTOS trial was the conception of decades’ worth of laboratory data that demonstrated that inflammatory pathways serve as crucial mediators for which traditional risks could exert their pathogenicity in atherosclerotic plaque development. (62) Canakinumab was administered quarterly to patients with a prior history of MI and, despite optimal stand-of-care pharmacotherapeutics, demonstrated a residual inflammatory risk marked by a CRP measure above 2 mg/L. (13) Participants showed a 15% reduction in 5-year mortality from nonfatal stroke, nonfatal MI, and mortality from CVD compared to placebo patients. (13) In individuals treated with 300 mg Canakinumab as a single subcutaneous injection every three months, CRP levels decreased by 60% from baseline, representing a 41% greater reduction than those receiving placebo. (13) Moreover, primary endpoint measures were reduced by 26% in those that achieved a greater than mean reduction in CRP. (13) Post hoc, the concentrations of IL-6 and IL-18 were compared in the Canakinumab group, where 300 mg at three months was associated with a 43.2% reduction in IL-6 at baseline but not IL-18. (22) For each tertile increase in IL-6 measured within those three months, the risk of MACE increased by 42%. (22) This was suggestive of the potential benefit of downstream neutralisation of IL-6 as a secondary messenger of IL-1 β and IL-18. As mentioned above, IL-6 neutralisation with 30 mg Ziltivekimab subcutaneously administered once

a month reduced CRP by 92% compared to placebo. (44) In a subsequent trial, ZEUS was established to evaluate the long-term cardiovascular outcomes in patients treated with Ziltivekimab in patients with chronic kidney disease, atherosclerosis and persistent inflammation. (45) While Canakinumab was not approved for use in participants with residual inflammatory risk and ASCVD, the trial provided essential evidence for the contribution of inflammation independent of other traditional risk factors. Specifically, the trial has inspired subsequent anti-inflammatory outcome trials to achieve greater reductions in cardiovascular event rates.

Colchicine

As a broad-spectrum anti-inflammatory with a relatively reduced price tag compared to Canakinumab, Colchicine, in patients with ASCVD, has been evaluated in two independent cohorts. (60,61) Colchicine has served as a standard-of-care therapy for the management of gout and is efficacious for the treatment of pericarditis. (125,126) The COLCOT evaluated the effectiveness of daily 0.5 mg oral Colchicine in reducing recurrent CVD events. (60) The population selected included those with a history of MI within 30 days since the recurrent risk of CVD events in this population is high. (60) COLCOT participants demonstrated a 22% reduction in primary endpoints characterised by mortality from CVD, nonfatal MI, nonfatal stroke, resuscitated cardiac arrest, and urgent hospitalisation for angina leading to revascularisation. (60) These results were driven by a 74% reduction in stroke and a 50% reduction in urgent hospitalisation for angina leading to coronary revascularisation. (60) Other primary endpoints were less affected, while reduced in the Colchicine-treated group. (60) Notably, within this study, an overall reduction in CRP was observed in both trial groups six months after MI. However, Colchicine treatment alone did not significantly diminish CRP concentrations. (60) Similarly to Canakinumab, Colchicine treatment resulted in a small but significant elevation in the risk of infection. Colchicine was subsequently tested in a population with stable CAD in LoDoCo2. Given the substantial reduction in CVD events, irrespective of a reduction in CRP, an alternative mechanism of Colchicine action independent of inflammation might be suggested.

Colchicine is described as an anti-mitotic drug with a mechanism of action on soluble tubulin, a significant component of the cytoskeleton. (127,128) While Colchicine is hypothesised to act on NLRP3 to reduce inflammation, (129) the concentrations required to achieve NLRP3 inhibition could not be reached within safety margins. (130) A recent study demonstrated that the anti-inflammatory effects of Colchicine could result from GFD15 release from hepatocytes, suppressing myeloid cell activation through the action of SHP-1. (130) In the subsequent LoDoCo2 trial, participants with stable CAD showed a 31% reduction in primary endpoints on Colchicine treatment compared to placebo. (61) These trials continued to highlight the urgent need for effective pharmacotherapeutics that target inflammatory pathways for the treatment of CVD. In mid 2023, low-dose Colchicine was approved for use in patients for the management of inflammation in ASCVD, making it the first drug of its class approved to treat residual inflammatory risk. (131) However, Colchicine is limited in its clinical utility to patients with intact hepatic and renal function. (131) Colchicine has a substantial half-life, where the liver and kidneys are primarily responsible for the metabolism and clearance from plasma. (132) In patients with

chronic kidney disease, Colchicine should be avoided due to the accumulation of toxic concentrations in the population. (132) Although a lower dose might be appropriate, further trials in a cohort with ASCVD and chronic kidney disease has not been established. Akin to the CANTOS, participants treated with Colchicine had an increased prevalence of severe infections, with the incidence of pneumonia increased by 57.1%. (60) Comparatively, however, there were no significant differences in the incidence of sepsis between treatment arms in the COLCOT trial. (60) Given these circumstances, an opportunity remains to develop novel pharmacotherapeutics that can target maladaptive inflammation without interference with host defence immunity.

Methotrexate (MTX)

The interest in inexpensive and broad-spectrum anti-inflammatories resulted in a subsequent clinical investigation into MTX for managing CAD. (133) MTX, prescribed for the treatment of RA, is proposed to reduce inflammation through various mechanisms. (134) From these mechanisms, de novo synthesis of purines and pyrimidines required for synthesising RNA and DNA that promote the rapid proliferation of cells, particularly leukocytes, is recognised. (134) Besides this pathway, MTX is hypothesised to act on dihydrofolate reductase to diminish the production of polyamines that promote ammonia and H₂O₂ production. (135) The Cardiovascular Inflammation Reduction Trial evaluated the anti-inflammatory benefit of MTX in a cohort with stable CAD. (133) This trial was the first among the cardiovascular outcome studies in which patients did not demonstrate any clinical benefit from treatment. (133) Particularly, the incidence of primary endpoints was not significantly different between MTX and placebo treatment. (133) From an inflammation perspective, MTX treatment was ineffective in reducing biomarkers IL-6, IL-1 β , and CRP despite significantly reducing white cell count. (133) Under these circumstances, this trial was terminated early due to inadequate efficacy in treating the population, together with an increased prevalence of adverse events such as non-basal skin cancer and infection. (61)

The P2X7R

Structure

The P2X purinoceptors are trimeric, non-selective cation channels that are activated by ATP. (136–138) In mammals, seven distinct P2X receptors are known, designated P2X1-P2X7, which share moderate sequence and structural homology. (136,137,139) Notably, each of the P2X receptors can form heteromeric assemblies in vitro, with the exception of P2X7R, which fails to interact with other P2X subunits. (140) The human *P2RX7* gene comprises 13 exons found on chromosome 12a24.31 close to the coding region of the P2X purinoceptor 4 (*P2RX4*). (141) The receptor is suggested to have originated by methods of gene duplication from the *P2X4R*, evidenced by the close sequence homology between the two. (142) The human *P2RX7* gene encodes for a 595 amino acid protein, where each receptor subunit is composed of three domains, including the two hydrophobic transmembrane domains (TMD), an intracellular carboxyl (C-) and N-termini, and a glycosylated extracellular loop containing the site for ATP to bind (143) Based on the crystal reconstruction from the panda (*Ailuropoda melanoleuca*) P2X4R, P2X7R, is predicted to resemble a dolphin-like shape, with the ectodomains akin to the

head and body, as the TMD are to the fluke (**Figure 3**). (144) The P2X7R is also differentiated from other P2X receptors due to the elongation of the C-termini characterised by an additional 200 amino acid residues. (145) The truncation of residues in the P2X7R C-terminus results in substantial deficits in the potency of BzATP at the orthosteric site. (146) The extracellular loops, referred to as the ectodomains, contain the residues for orthosteric and allosteric binding sites. (139,147)

While other P2X receptors are activated by nanomolar concentrations of ATP (half-maximal effective concentration, $EC_{50} = 1\text{-}10\text{ }\mu\text{M}$) (148,149), the P2X7R requires higher concentrations of endogenous ATP ($EC_{50} = 0.5\text{-}1\text{ mM}$) for activation (150). Although not yet confirmed, the relatively low affinity of ATP at the P2X7R might be attributable to the accessibility of the orthosteric pocket to ATP. (151) Evidence from cryoelectron microscopy indicates that the ATP-binding pocket is narrow, which might influence the time it takes for ATP to bind to this region. Moreover, the P2X7R is not susceptible to desensitisation, as seen for other P2X purinoceptors. (152) Within P2X pharmacology, the desensitisation process limits the action of ATP on the receptor. Under these conditions, receptor activation could persist in the presence of a constant stimulus, while other P2X receptors return to basal conditions within seconds to minutes. (152) Structure studies determined that the P2X7R contains a 'C-C anchor' that created a physical link between the 'cytoplasmic cap' and the TMD2, (151) permanently stabilising the cytoplasmic cap. Permanent stabilisation of the cytoplasmic cap to the TMD2 is achieved through palmitoylation of 5 residues C362, C363, C374, C377, and S360 on the C-C anchor. (151) In contrast, another P2X purinoceptor, the P2X3R desensitises within seconds and has a cytoplasmic cap that only persists in the ATP-bound open state. (153) Unravelling the structural properties of the P2X7R has provided valuable insights into the molecular principles governing the receptors' pharmacology and contribution to disease.

Function

As mentioned in the previous section, the P2X purinoceptors are transmembrane ion channels activated in response to extracellular ATP. (141) This receptor plays an important role in the maintenance of cellular homeostasis through the modulation of sodium, calcium, and potassium concentrations to facilitate physiological processes such as neurotransmission and cardiomyocyte contraction. (48,154–156) Moreover, the P2X7R modulates mitochondrial metabolism and ATP synthesis, cell growth, and differentiation under the instructions of ATP. (157) However, its relevance to inflammation has garnered the receptor significant attention. (158–160) The P2X7R is implicated in several pathological conditions, such as CVD, neuropsychiatric and neurodegenerative diseases, RA, and gastrointestinal disorders. (161–165) The role of the receptor, ascribed to its high prevalence on immune cells in the central nervous system (CNS) and peripheral tissue, is described as an 'alarmin' sensor that responds to 'danger' via the activation of various signalling cascades that provoke the maturation and release of cytokines (166–168). To a lesser degree, the P2X7R is expressed on non-hematopoietic cells such as fibroblasts and smooth muscle cells, where the function is less well characterised. P2X7R expression on fibroblasts is associated with the production of microvesicles from the Golgi compartments

and secretion of IL-1 β and subsequent IL-6. (169,170) Under these circumstances, the P2X7R function is understood to be cell-type specific and dependent on a range of experimental factors. This includes the presence of genetic polymorphisms, isoform variants, stimulus intensity, and the availability of ions that can act as negative allosteric modulators. (146,147,171,172) Nonetheless, its function from an immunobiological standpoint has made it a target of diagnostic and therapeutic interest in inflammatory disorders.

Surprenant et al. 1996 cloned and characterised the P2X7R based on the potency of ATP at the receptor. (173) It is now understood that sustained activation of the P2X7R can elicit a lytic function in cells through the assembly of the NLRP3 inflammasome, (174) a multiprotein complex that consists of the NLRP3 protein, apoptosis-associated speck-like protein containing CARD domain (ASC), and procaspase-1 (**Figure 4**). (175) The activation of P2X7R promotes the formation of a macropore, identified as the expansion of the transmembrane domain region. (144) The NLRP3 inflammasome is essential for the cleavage of precursor caspase-1 into active caspase-1, an enzyme that cleaves biologically inactive cytokines into their active form. (176) Caspase-1 has also been identified to cleave the N- and C-terminal domains of GSDMD, where the N-terminal fragment translocates to the membrane surface and forms large pores with an internal diameter between 13-34 nm. (31,176) The formation of these pores promotes the secretion of cytokines from intracellular compartments. (177–179) However, other mechanisms independent of P2X7R can also promote cytokine secretion. Given that these pores contribute to the disruption of the electrochemical gradient and the influx of water leading to cellular rupture, the consequence of P2X7R activation is the initiation of pyroptosis. (180) Aside from the formation of GSDMD pores, activation of caspase-1 is also suggested to initiate apoptosis through caspase-3-dependent mechanisms, which is relevant in cells with low GSDMD expression. (31) Under these circumstances, dying cells release DAMPs such as ATP, heat shock proteins, and IL-1 β . (181) These DAMPs contribute to the sustained activation of P2X7R, further promoting the continuation of this response without consolidation. This process facilitates chronic tissue-specific inflammation due to extensive cellular damage. (163–165,182,183) For this reason, for the last two decades, the P2X7R has been highlighted as an attractive therapeutic target for inflammatory diseases.

Single nucleotide polymorphisms (SNPs) of the P2X7R

In the context of non-hereditary disease, SNPs can confer unique genetic-based insights into a populations vulnerability or resilience to certain diseases. (184–186) For diseases that do not carry an overt inheritance pattern, as is the case for hereditary hypercholesterolemia and some hypertrophic cardiomyopathies, SNPs may help to disentangle the risk of an individual. (187,188) Instead, SNPs are marked by single base mutations that can occur within genes or regulatory regions. (189) Mutations are either characterised as non-synonymous, meaning an amino acid is modified, resulting in structural changes to the protein, or synonymous, which assumes a ‘silent’ effect. (190) The *P2RX7* is highly polymorphic, with at least 28 non-synonymous SNP variants identified. Of these 28 SNPs, 15 are characterised as either gain- or loss-of-function mutations. (191) SNPs T357S, E496A, and I568N, located within the region encoding the C-terminus, have been associated with significant impairments to macropore formation (**Table 2**). (192–194) Particularly, substitution of a glutamic acid for alanine

at amino acid residue 496, lead to substantial impairments in P2X7R-mediated apoptosis in T cell populations in homozygous carriers. (193). Moreover, smokers who were homozygous for the minor allele exhibited a reduced risk of ischemic stroke, suggesting that a reduction of P2X7R function may offer protective benefits in ischemic stroke. (195) Comparatively, the loss-of-function of I568N was associated with reduced cell surface expression, indicating the importance of this region in the translocation of P2X7R to the cell surface. (194) The role of Q460R variant, which is also contained in the C-terminus region, is more controversial. (196) Despite its potential association with mood disorders such as bipolar and depression, three independent studies found no significant impact of Q460R on receptor activity, (196–198) whereas one study determined it could be a gain-of-function variant. (199) However, co-expression of the Q460R mutation with another variant might explain this discrepancy, given that a previous study determined that heterogenous expression of Q460R and the wild-type P2X7R resulted in reduced activity. (200)

Mutations V76A and R276H are present in the ectodomain and confer a loss-of-function in P2X7R functions, likely related to ATP-receptor interaction. (191,198) Previous studies implicated the C minor allele in SNP V76A with a reduced incidence of multiple sclerosis. (201) In contrast, the T allele increased the frequency of multiple sclerosis. (201) Patients carrying the T allele exhibited increased cytosolic pore formation in response to BzATP. (201) In patients with gout, the T allele was associated with a higher prevalence of gout onset and heightened IL-1 β release from leukocytes than mutant carriers. (202) Comparatively, R276H was associated with the onset of gout, but no difference was observed in IL-1 β concentration (202), although further studies are required to characterise this variation. Of those mutations from the ectodomain, H155Y and R270H confer gain-of-function mutations. (198) Amongst these variants, the capacity for ethidium uptake and IL-1 β release was substantially elevated compared to the wild-type variant. Intriguingly, however, the co-expression of these variants markedly reduced the amplitude and time for pore formation as opposed to when expressed alone. The co-expression of another gain-of-function SNP, A348T, encoding for the TMD2, preserved this response. (198) Since these mutations are present within exon regions, structural alternations in the protein might cumulatively affect the function of P2X7R. Although not a *P2RX7* SNP, the downstream multiprotein complex NLRP3 that P2X7R acts upon also carries a distinct gain-of-function polymorphism in the intron region that increases the expression of *NLRP3*. (57) This mutation results in an increase in systemic inflammation, which is associated with a greater risk of CAD (**Table 2**). (57) Given the diverse and potentially deleterious polymorphisms associated with the *P2RX7* and its downstream targets, direct inhibition of its activation in disease states remains an attractive therapeutic strategy.

Table 1. Overview of cardiovascular outcome trials evaluating anti-inflammatory drugs in patients with ASCVD.

Study	Drug	Target	Cohort	Result	Citation
JUPITER	Rosuvastatin	hsCRP, LCL-C	Healthy population	50% reduction in LDL-C concentration in a population with normal LDL-C and no history of CVD. Primary endpoints indicated by first incidence of nonfatal MI, nonfatal stroke, hospitalization for unstable angina, arterial revascularization, or mortality from CVD was reduced by 50%. Participants allocated to rosuvastatin demonstrated a 37% reduction in hsCRP.	12
CANTOS	Canakinumab	IL-1 β neutralisation, hsCRP	ASCVD, recurrent residual risk	Reduced primary endpoints of nonfatal MI, nonfatal stroke, and death from CVD by 15%. Reduced hsCRP concentration by 39%. Secondary endpoints, indicated by primary endpoints, including hospitalization from unstable angina requiring unplanned revascularization reduced by 17%.	13, 14
COLCOT	Colchicine	NLRP3/Neutrophil chemotaxis – alternative hypothesis myeloid cell regulation through GFD15 action of SHP-1	ASCVD, recurrent residual risk	Reduced primary endpoints of mortality from CVD, nonfatal MI, nonfatal stroke, resuscitated cardiac arrest, and urgent hospitalization for angina leading to revascularisation by 22%. Reduction in secondary endpoints by 15%. No significant reduction in hsCRP measures between groups.	60
LoDoCo2	Colchicine	NLRP3/Neutrophil chemotaxis – alternative hypothesis myeloid cell regulation through GFD15 action of SHP-1	ASCVD; stable cohort	31% reduction in primary endpoints indicated by CVD mortality, nonfatal MI, nonfatal stroke, and unstable angina requiring revascularization. Secondary endpoints, marked by CVD mortality, nonfatal MI, and nonfatal stroke reduced by 28%. Measures of hsCRP not reported	61
CIRT	Methotrexate	Synthesis of purines and pyrimidines	ASCVD, metabolic disease	No reduction observed in primary endpoints indicated by CVD mortality, nonfatal MI, nonfatal stroke, and unstable angina requiring revascularization. No significant difference in hsCRP, IL-6, and IL-1 β concentrations compared to placebo.	133
RESCUE	Zilivertimab	IL-6 neutralisation, hsCRP	Chronic kidney disease; residual inflammation associated with ASCVD risk	30-mg zilivertimab reduced hsCRP concentration by 92% in 12-weeks. Significant reduction in IL-6 concentration across cohort. Trial canceled due to COVID-19. Subsequent trial ZEUS commenced to evaluate IL-6 neutralisation to reduce cardiovascular event rates.	44

Splice variants of the P2X7R protein

Common isoforms of the human P2X7R give rise to further diversity amongst P2X7R expression in disease. In humans, ten common isoforms arise from the *P2RX7* gene referred to as P2X7RA to P2X7J, with P2X7RA characterised as the canonical full-length receptor (**Table 3**). (203) The expression of the full-length receptor is unsurprisingly abundant across peripheral blood mononuclear cells (PBMCs) populations. (146) The addition of a premature stop codon between exons 10 and 11 resulted in the truncated P2X7RB isoform. (146) Specifically, the isoform is characterised by the removal of 231 amino acid residues attributed to the C-terminus region and modification to 18 amino acids that proceed to the TMD. (146) The result is the synthesis of a protein demonstrating a 4.6-fold reduction in potency at the orthosteric site and a diminished capability for ATP-induced macropore formation. (146) However, antagonists were determined to retain activity across isoforms, indicating that the allosteric site is preserved. (146) While the rat crystal structure represented the P2X7R as a homotrimer conformation, in humans, co-expression of P2X7RA and P2X7RB isoforms were also found to assemble into heterotrimers with a 1:2 or 2:1 configuration. (204) The activity of the co-assembled heterotrimer results in a receptor with heightened calcium mobilisation and pore-forming capabilities, indicative of increased receptor activity. (204) This is particularly relevant as P2X7RB is abundantly expressed in lymphocytes compared to P2X7RA, although further studies are required to understand the prevalence of the heterodimer and its association and function in disease. As mentioned above, the C-terminus is important for P2X7R function, particularly in relation to macropore formation and receptor translocation. (194,205) However, less well-known is the crucial role of the C-terminus in facilitating intracellular signalling through interaction with structural proteins via the phosphorylation of tyrosine. (206)

The involvement of at least 11 identified proteins is shown to be important in the regulation of the cytoskeleton and potentially facilitate membrane 'blebbing' (206), a P2X7R-dependent process that leads to apoptosis via alterations to cellular architecture. Isoforms P2X7RC, P2X7RE, and P2X7G also demonstrate the same stop codons between exons 10 and 11 combined, deleting specific exons in each conformation (**See Figure 5**). (146) The consequence and dispersity across tissues of these isoforms are less well-understood. However, considering the deletion of the C-terminus and the removal of exons encoding the ectodomain in isoforms P2X7RC and P2X7RE, it is hypothesised that these isoforms are non-functional receptors. Isoforms P2X7RG and P2X7H result from the spontaneous insertion of the 'N3' exon between exons 2 and 3 that introduces a start codon, leading to the translation of a protein without the TMD. (207) P2X7RH resulted in a protein that was unresponsive to BzATP treatment. (146) This isoform is associated with poor calcium mobilisation and pore-forming capabilities in response to BzATP activation. (146) Comparatively, to isoform P2X7RB, the expression of P2X7RH was low amongst tissues and virtually absent in PBMCs. (146) Isoform P2X7RJ results from the frameshift mutation of exon 8, which translates to a protein of only 258 amino acid residues. (207) The consequence is a protein without the C-terminus, TMD2, and one-third of the ectodomain. As expected, the protein was ineffective at promoting pore formation and, thus, apoptosis after BzATP stimulation. (207) Comparably to the heterotrimeric configuration of P2X7RA and P2X7RB isoforms, the co-expression of P2X7RA and P2X7RJ culminated in another

oligomerised configuration. (207) This heterotrimeric structure rendered the P2X7RA configuration useless in apoptosis. In rodents, which are commonly used for in vivo studies, the P2X7R isoforms P2X7RA through to P2X7RD are known. (208) Additionally, the P2X7RK isoform, results from the inclusion of an additional exon between exons 1 and 2, was found to be unique to rodents. (209) This isoform is characterised by modifications to the N-terminus and transmembrane domain 1. Its function reflects this modification, where a slower desensitisation time and increased sensitivity for BzATP results in increased ethidium uptake, suggesting its role as a potential 'death' receptor. (209)

Post-translational modifications associated with the P2X7R function

The C-terminus was hypothesised to govern the desensitisation kinetics of P2X purinoceptors for over two decades before the publication of the rat P2X7R. (151,153,210–212) Structural studies using the human P2X73R identified a structure referred to as the 'cytoplasmic cap' visible in the activated state that was absent in the apo (close) state. (153) The structure for the P2X7R demonstrated that this region was fixed, indicated by the visibility of the structure in the apo and open states. This study suggested a permanent structural 'glue' that could hold the cytoplasmic cap in place, preventing desensitisation after activation. (151) This glue was recognised as the 'C-C anchor', termed based on the cysteine-rich region that could physically link the cap to the TMD2. (151,213) The stabilisation of this structure is owed to the discovery of palmitoylated cytosine residues, together with one serine that restricts the recoil of the cytoplasmic cap. (151) As mentioned in the previous two sections, mutations or deletions in the C-terminus affect the opening of the macropore. (146) Moreover, glycosylation of five asparagine residues in the ectodomain regulates receptor translocation and sensitivity for ATP. (214) Specifically, the N-linked glycosylation of these residues, particularly N187, was associated with effective receptor translocation to the cell surface. Mutation of this residue resulted in a substantial decline in BzATP-mediated pore formation. (214) However, this may be an artifact of low expression due to a decline in receptor trafficking. In inflammation, post-translational modifications of the cytoplasmic and ectodomains are significant for the unique kinetic properties governing P2X7R pharmacology

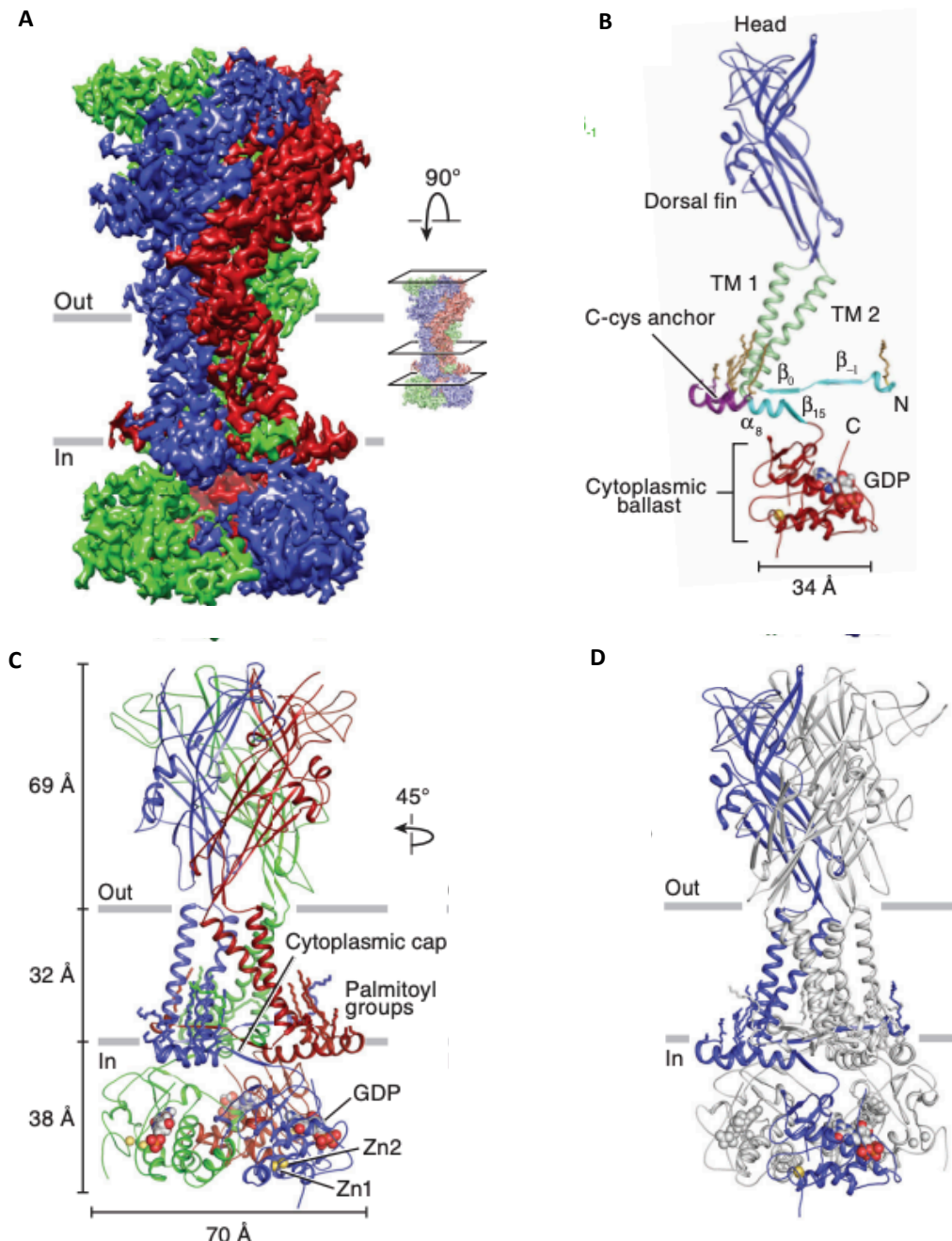


Figure 3: Overall Structure of the Full-Length rat P2X7R in the open state. **A)** Three-dimensional reconstruction of the full-length rat P2X7R in the ATP-bound open state, **B)** Ribbon representation of a single subunit highlighting the domains with their colloquial names, **C)** Ribbon representation of the trimeric P2X7R structure labelled in the apo (closed) state, **D)** Ribbon representation representing the interaction between to protomers of the P2X7R. **Adapted from** (151). Copyright Elsevier.

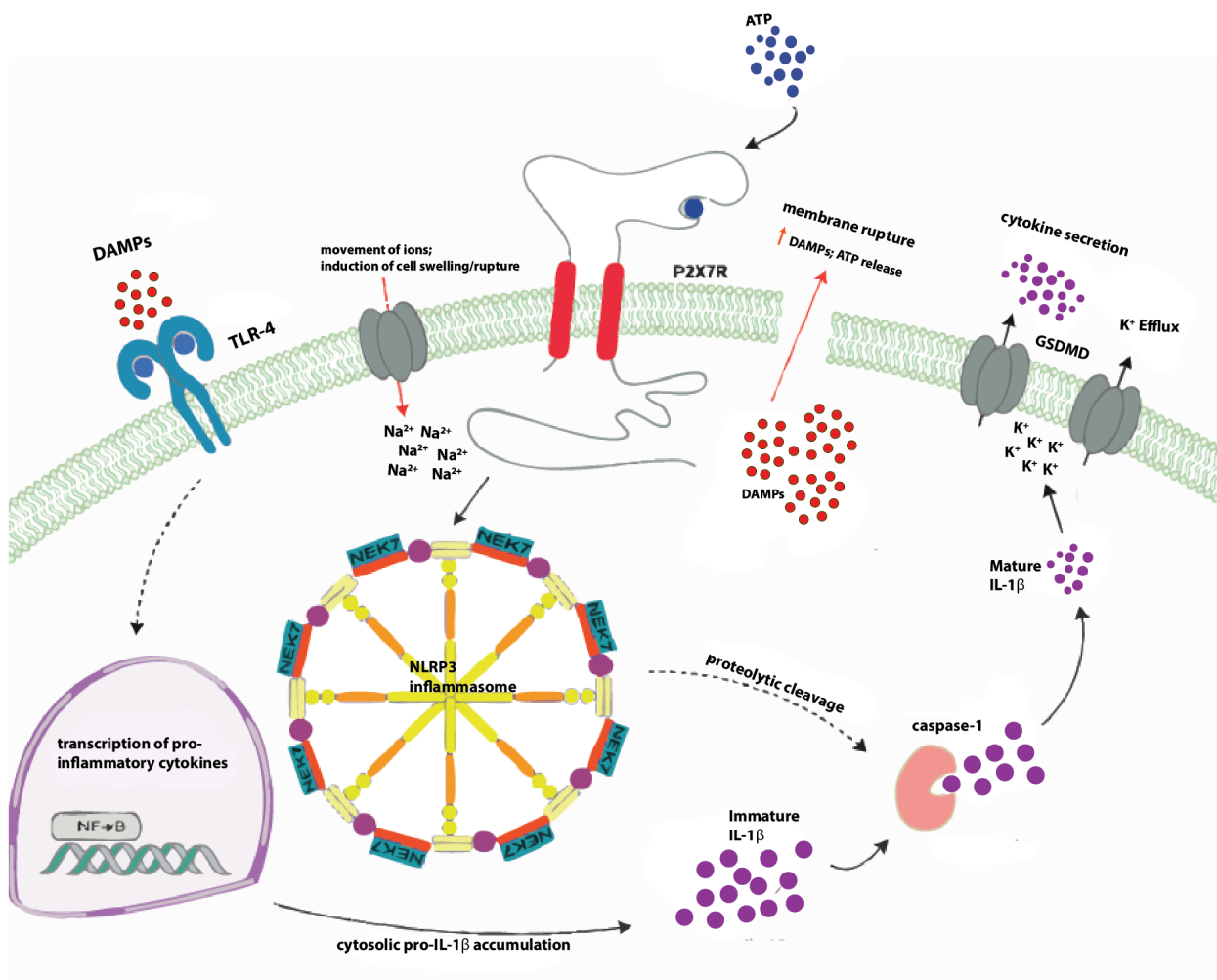


Figure 4. Simplified mechanism of P2X7R activation. The toll-like receptor-4 is activated by DAMPs. This results in downstream activation of NF- κ B. Activating this transcription factor results in the synthesis of precursor cytokines such as IL-1. These cytokines are inactive and primarily reside in the cytosol. The P2X7R is activated by accumulating ATP released from injured or dying cells. Activation of the receptor results in downstream assembly of the NLRP3 inflammasome, a multimeric complex that cleaves pro-caspase-1 into caspase-1. Caspase-1 processes precursor cytokines into their mature, where the active cytokine has a role in systemic inflammation. Caspase-1 also cleaves GSDMD between the C- and the N-terminus, where the N-terminus fragment is translocated to the cell surface to promote the secretion of inflammatory cytokines. The cell begins to swell, with potassium released, which promotes cellular rupture and release of cytokines and DAMPs. Adapted from (183). **Created in Adobe Illustrator.**

Variations in P2X7R structure and function across species

There are notable differences in the structural and kinetic properties associated with the human and rodent receptors. Despite these variances, protein alignment shows a close conservation of the P2X7R across species despite key amino acid residue modifications noted (**Figure 6A**). The mouse P2rx7 gene is located on chromosome 5 (NC_000071.7), within a region that shows conserved synteny with the corresponding human gene (Figure 6A-B). (203) The full-length P2X7RA isoform in C57BL/6J mice harboured a single residue change from proline to leucine at position 451 (P451L) compared to BALB/c mice. (174) This mutation maps the C-

terminus domain containing the TNF Receptor 1-death domain and the SH3 Domain Binding Protein 1, responsible for pore formation and protein interaction, respectively. (148) This modification reduces lytic activities, thus underestimating the contribution of the receptor to inflammation. From a pharmacological perspective, BzATP was 10-fold less potent at the mouse receptor than the human receptor. (215) These apparent differences could be explained by changes to the C-terminus affecting pore formation in mice P2X7R. Key residue variances in the ectodomain could also explain intrinsic differences between mouse and human receptors. Previous studies have aimed to address why P2X7R demonstrates such low potency for ATP compared to other receptors. Reconstruction of the rat P2X7R and the human P2X3R were used to explore this relationship. (151,153) However, between rats and humans, there are fewer species differences in the potency of ATP and its antagonists at the P2X7R, when compared with mice.

The orthosteric site harbours small residue changes with nonpolar or hydrogen additions in the P2X3R. (151,153). However, these modifications are suggested to account for up to a 10-fold difference in potency. Differences in potency are instead attributed to a concept known as 'pocket breathing', which is based on protein flexibility and associated with the narrowing of the orthosteric pocket. (151) This would decrease the affinity for ATP, based on the time required for ATP to reach the pocket at the P2X7R. (151) Further studies are required to evaluate whether these modifications at the mouse receptor could explain these inherent differences. As expected, pharmacological variance in P2X7R across species has complicated preclinical assessment. Previous studies demonstrated that the P2X7R antagonist A-740003, used in preclinical studies, show greater activity at human and rat receptors than the mouse receptor. (215) The same is true for most common inhibitors, such as pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid and MRS2159, where Brilliant Blue G and KN-62 lose potency completely at mouse receptors. (215) This inherent difference in potency across species may depend on the mode of action at the receptor, referring to allosteric or orthosteric inhibition, although further studies are required. Interspecies differences must be assessed before the commencement of preclinical studies to understand the potential advantages and limitations of experiments targeting P2X7R biology.

The P2X7R as a therapeutic target in CVD

Atherosclerosis

Current evidence for the involvement of P2X7R-mediated inflammatory activity in atherosclerosis remains sparse. Historically, as demonstrated below, the receptor garnered significant interest in neurodegenerative conditions due to the abundance of P2X7R on microglia in the CNS. (183,216,217) The expression of P2X7R in peripheral cells is primarily localised to the surface of monocytes, where its activation is associated with the release of cytokines. (175) From the perspective of vascular disease, treatment with the P2X7R antagonist A438079 in a murine model of sepsis reduced leukocyte adhesion to endothelial cells in the brain microvasculature. (218) This result was confirmed in a separate cohort of mice, where P2X7R inhibition with a short hairpin RNA specific to the P2X7R reduced leukocyte adhesion in brain microvasculature and the

expression of adhesion molecule ICAM-1. (218) Under low mechanical shear stress, a feature of narrowed vessels in atherosclerosis, the P2X7R is known to be upregulated on endothelial cells in vitro and in vivo. (219) Of note, P2X7R expression was increased in atheroprone regions in the mouse aorta, where the expression of CD39 was downregulated. (219) Given the crucial role of CD39 as a regulator of ATP degradation, (220) its downregulation is associated with ATP accumulation and the activation of the P2X7R. This study links the potential association between P2X7R expression under low shear stress conditions and the upregulation of adhesion molecules, such as ICAM-1, necessary for leukocyte transmigration in early atherosclerosis. (218,219) From an immune cell perspective, our team recently demonstrated that PBMCs from patients with ASCVD demonstrate heightened BzATP-induced IL-1 β release compared to patients with no CAD. (221) We confirmed that this response was mediated through the P2X7R via co-incubation of PKT100 in the presence of BzATP. (207) We showed that PKT100 reduced IL-1 β levels from PBMCs to near baseline concentrations, confirming a role for P2X7R in downstream IL-1 β release.

Previous evaluations in a murine atherosclerosis model showed that atherosclerotic lesions in the aortic arch of LDL-receptor (LDL-R)^{-/-}, P2X7R^{-/-} mice were reduced compared to LDL-R^{-/-}, P2X7R^{+/+} mice. (222) This was associated with a significant reduction in macrophages and intraplaque lipid-rich atherosclerotic lesions. (222) Moreover, the expression of P2X7R in human atherosclerotic lesions from carotid endarterectomy was upregulated compared to control vessels. (222) ApoE^{-/-} mice treated with a small interfering RNA against the P2X7R were shown to have reduce lesion formation in aortic sinus segments. (223) Mice in this study also demonstrated substantial reductions in IL-1 β concentration in plasma. (223) Human unstable atherosclerotic plaques showed higher *P2RX7* gene expression compared to stable vessels. (224) This was also associated with heightened downstream NLRP3 and IL-1 β expression, where IL-1 β protein from plaque lysates increased in unstable lesions. (224) The heightened expression of IL-1 β in protein lysates was reduced after ex vivo incubation with specific P2X7R antagonist A740003. (224) This also resulted in the suppression of matrix metalloproteinase-9 (MMP-9), an important enzyme involved in matrix degradation and plaque destabilisation. (224) This mechanism was demonstrated in a previous study, where ATP stimulation increased MMP-9 concentrations in supernatants of PBMC-derived monocytes. (225) Treatment with KN62, a P2X7R antagonist, reduced MMP-9 levels, (225) suggesting that P2X7R plays a regulatory role in MMP-9 release. Furthermore, the expression of P2X7R was close to luminal thrombi and inflammatory infiltrates, co-localised to the endothelium and CD68⁺ macrophages. (226) While further evidence is required to increase the certainty of P2X7R as a therapeutic target in atherosclerosis, its downstream targets, NLRP3 and IL-1 β , have been demonstrated to be strongly implicated in ASCVD. This is particularly evident from the CANTOS trial, where the neutralisation of IL-1 β was associated with improved clinical outcomes. (13)

MI

Inflammation, in the context of MI, is associated with rapid infiltration of neutrophils, followed by macrophages on days 3 to 6. (227) The increase in caspase-1 within minutes of ischemic injury suggests the pivotal role of the

inflammasome in myocardial repair after infarction. (94) In a murine model of MI, inhibition of P2X7R expression with a siRNA inhibitor was shown to preserve heart function. (94) Mice treated with a non-competitive P2X7R antagonist, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid, in the same mouse model demonstrated reduced infarct size and cell death in border regions measured by Terminal deoxynucleotidyl transferase dUTP nick-end labeling. (94) The relationship between P2X7R inhibition and inflammation-mediated cytokine release was not explored, but these results indicate protection against inflammation-mediated cell death. This is supported by studies in ASC^{-/-} mice where downstream deletion of ASC, a component of the NLRP3 inflammasome, is associated with substantial reductions in neutrophils and macrophage infiltration in a murine ischemia-reperfusion MI model. (228) Changes in protein expression of key inflammatory modulators such as IL-1 β , IL-6, and MCP-1 were decreased in ASC^{-/-} mice hearts after MI. (228) Rats that underwent MI showed strong expression of P2X7R on macrophages in the ischemic region between days 1 to 7. (229) Macrophages in these early stages express a proinflammatory phenotype that is proposed to activate pro-fibrotic pathways. (230) Based on the observed effects of IL-1 β on adverse remodelling after MI, the neutralisation of IL-1 β activity after an ischemic event has been evaluated. (231)

The effectiveness of a P2X7R antagonist in IL-1 β reduction was also compared with Anakinra, an IL-1 receptor antagonist in rats. (229) After MI, infarct size was reduced in A-740003 and Anakinra were associated with less macrophage infiltration. (229) Comparatively, Anakinra in combination with A-740003 was not associated with better outcomes compared to Anakinra or A-740003 alone. (229) Previous clinical studies have shown similar outcomes, where patients receiving traditional anti-inflammatory treatments, such as MTX or sulfasalazine in combination with AZD9056, did not experience any additional benefit beyond their current therapies. (232) This provides reasonable evidence that a P2X7R antagonist in combination with a traditional anti-inflammatory agent may not reduce the magnitude of inflammation. While preclinical evidence substantiates the involvement of P2X7R in inflammation associated with MI, human studies are more sporadic. An expected loss of function mutation rs3751143 was associated with a reduced risk of MI in smokers. (195) This SNP, with a minor allele frequency of 20%, is associated with a loss-of-function mutation. (193) Moreover, *P2RX7* expression was shown to be upregulated in PBMCs from patients with MI and was associated with CRP concentration. (233) Expression levels of *P2RX7* were also associated with an increased risk of CVD events within 360 days, (233) demonstrating its potential prognostic value. While preliminary evidence suggests the potential significance of P2X7R in MI, further evidence is required to understand the importance of this receptor as a diagnostic target in ASCVD.

Table 2. Common single nucleotide polymorphism effecting the P2X7R and downstream NLRP3 inflammasome

SNP	Referenced abbreviation	BP	AA Substitution	GRCh38.p13	MAF (HapMap)	MAF(1000Genome)	Effect	Citation
Rs17525809 NM_002562.6:c.227T>C	V76A	253	Val76Al	chr12:12115488 6	C=0.062 (82/1318)	C=0.049 (250/5008)	Partial loss of function, reduced calcium permeability, decreased ATP-induced ethidium uptake and IL-1 β release in PBMCs.	201, 202
Rs208294 NC_000012.12:g.121162 450T>A	H155Y	489	His155Tyr	chr12:12116245 0	T=0.471 (792/1680)	T=0.470 (2354/5008)	Gain-of-function, increased calcium permeability, heightened ethidium uptake upon ATP stimulation.	197, 198
Rs7958311 NC_000012.12:g.121167 552G>A	R270H	835	Arg270His	chr12:12116755 2	A=0.260 (442/1700)	A= 0.256 (1281/5008)	Gain of function, increased and sustained ethidium uptake in response to ATP.	198
Rs7958316 NC_000012.12:g.121167 570G>A	R276H	853	Arg276His	chr12:12116757 0	A=0.021 (28/1330)	A=0.012 (62/5008)	Gain of function, increased calcium permeability, heightened ethidium uptake upon ATP stimulation.	201
Rs1718119 NM_002562.6:c.1042G>A	A348T	1068	Ala348Thr	chr12:12117730 0	A=0.362 (681/18820)	A=0.323 (1615/50080)	Gain of function, sustained ATP-activation and pore formation capabilities. Increase P2X7R function was co-expressed with other variants.	198
Rs2230911 NM_002562.6:c.1070C>G	T357S	1096	Thr357Ser	chr12:12117732 8	G=0.143 (47/328)	G=0.165 (826/5008)	Partial loss of function, ATP-induced ethidium uptake reduced.	192
Rs2230912 NM_002562.6:c.227T>C	Q460R	1405	Gln460Arg	chr12:12115488 6	C=0.062 (82/1318)	C=0.050 (250/5008)	Undetermined. Reported to have a neutral effect or potential gain of function in ATP-induced yo-pro-dye and ethidium uptake assays.	196, 199
Rs3751143 NM_002562.6:c.1513A>C	E496A	1513	Glu496Ala	chr12:121184.5 01	C= 0.195 (369/1890)	C= 0.208 (1039/5008)	Loss of function; ATP-induced ethidium uptake is abolished but the channel retains response to ATP.	193, 198
Rs1653624 NM_002562.6:c.1703T>A	I568N	1729	Ile568Asn	chr12:12118471 7	A=0.006 (2/326)	A=0.006 (39/6404)	Loss of function, reduced ATP-induced ethidium uptake due to failure of the receptor to translocate to the cell surface.	194
Rs10754558 NC_000001.11:g.247448 734G>C				chr1:247448734	G = 0.340 (111/326)	G = 0.361 (1735/5008)	Increased expression of the NLRP3 inflammasome increased IL-1 β production from PBMC-derived monocytes. Increased association in systemic inflammation marked by plasma IL-18 and CRP.	57

BP (Affected Base Pair): The specific nucleotide position where a mutation occurs.

AA Substitution (Amino Acid Substitution): Replacement of one amino acid with another due to a genetic variation.

MAF (Minor Allele Frequency): The frequency of the less common allele in a population.

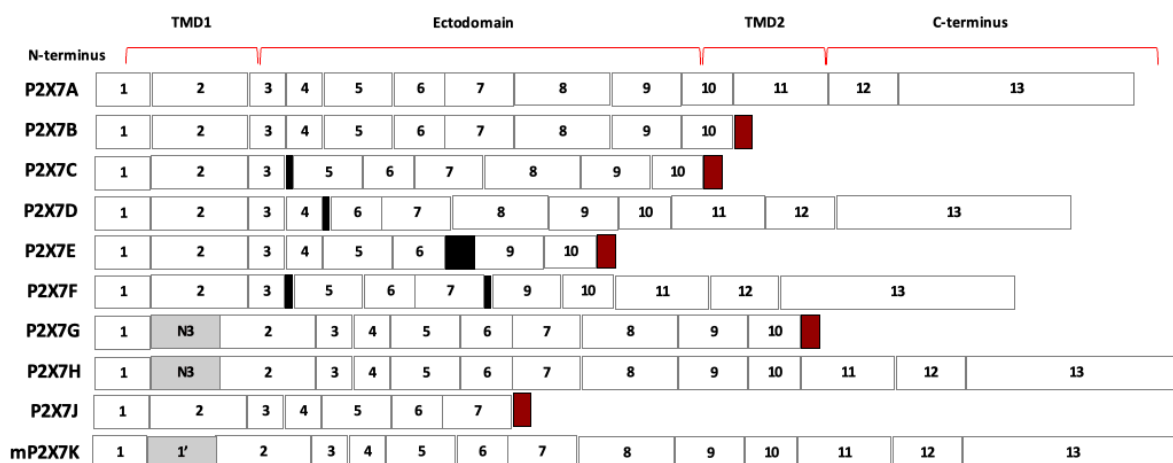


Figure 5: Representation of the exon arrangement for P2X7R isoforms. P2X7RA is the full-length canonical receptor marked with the regions encoded by exons. A dark red box marks the insertion of stop codons. A black box marks the deletion of an exon. A grey box marks the addition of a new exon. P2X7K has been exclusively identified in mice, marked by an m.

Clinical translation of P2X7R antagonists

AstraZeneca

RA

Anakinra, the IL-1 receptor antagonist, exhibited modest efficacy in participants with RA when administered through subcutaneous injections over 24 weeks. (234) Like Canakinumab, Anakinra was also evaluated in a cohort with a recent history of MI, where administration soon after the event was associated with better 1-year survival from HF. (13,235) Its use in RA elicits modest improvements in ACR20 scores, where targeting IL-1 alone may be insufficient to achieve positive trial endpoints. Compared to atherosclerosis, RA is a classic immunological disorder, where self-recognition of non-pathogenic cells in synovial joints as pathogenic drives cartilage degradation. (168,236) Under these circumstances, simultaneous inhibition of targets upstream of IL-1 could be therapeutically advantageous for treating RA. Given the substantial role of P2X7R in the modulation and release of these cytokines, AstraZeneca (AZ) synthesised a series of compounds to treat RA. AZD9056 was the first P2X7R antagonist to enter clinical development, following its synthesis by AZ in 2003. (237,238) The compound was part of the AZ adamantane amide series that showed strong selectivity for the P2X7R. (235) The initial lead, while potent, was demonstrated to have metabolic liabilities around the adamantane cage, resulting in its degradation. The addition of the hydroxypropylamino tail increased the stability of the compound, leading to its clinical translation. In vitro screening showed that AZD9056 diminished BzATP-induced IL-1 β and -18 release in lipopolysaccharide (LPS) primed human PBMCs. (236) In the context of RA, AZD9056 was demonstrated to reduce IL-1 β in human synovial cells potently. (236)

As highlighted above, a substantial limitation in evaluating P2X7R antagonists in vivo is due to the pharmacological variations between the rodent and the human receptors. For this reason, preclinical evaluations for AZD9056 were conducted with a rat-specific structural analog AZ11657312, as AZD9056 lacked

selectivity at the rodent receptors. (236) AZ1165731, delivered for 6 days at 30 or 60 mg/kg, effectively reduce inflammation in synovial membranes and sub-linings in a rodent arthritis model. (236) Additionally, rats demonstrated a substantial reduction in arthritic features such as chondronecrosis and mechanical hyperalgesia. (236) Radiographical assessment of the lesions demonstrated reductions to the articular tibiotarsal joint, (236) suggesting that AZD9056 was protective against inflammation-induced joint injury. After the onset of the disease, treatment with 60 mg/kg of AZ11657312 decreased inflammatory scores but did not improve arthritic features if treatment was delayed. (236) These results point to the limitations of P2X7R inhibition in RA, suggesting its potential role as a preventative rather than a reparative intervention. Under these conditions, AZD9056 entered phase I clinical trials, where it was established to be safe and generally well-tolerated in healthy participants who received 400 mg daily for up to 6 months. (1)

The positive outcomes of the phase I clinical trial resulted in a subsequent phase II clinical trial where the efficacy of AZD9056 was evaluated in a population with a recurrent history of unresolved RA despite receiving background MTX or Sulphasalazine. (232) These participants were labelled as extensively inflammatory, indicated by a CRP concentration of more than 10 mg/L. (232) In a 4-week pilot study, 65% of participants allocated to AZD9056 treatment achieved ACR20 compared to only 27% of participants allocated to the placebo. (232) ACR20, defined by an improvement of 20% in the number of tender and swollen joints. However, subsequent evaluation for 20 weeks demonstrated no improvement in ACR20 in the AZD9056 group compared with placebo. (232) Moreover, there was no systemic reduction in CRP in the AZD9056 group, even though ATP-induced IL-1 β from ex vivo patient-derived monocytes was reduced. (232) Considering that CRP is acutely synthesised in response to IL-6, it could be hypothesised that other mechanisms independent of IL-1 β may regulate this RA phenotype. Indeed, MTX and Sulphasalazine indirectly inhibit IL-1 β , although many of their mechanisms are unclear. Of interest to P2X7R, both compounds target the transcription factor NF- κ B, preventing the production of immature proinflammatory cytokines pro-IL-1 β and -18. (239,240) Under these assumptions, several common inflammatory pathways suppressed by MTX do, however, correspond with the P2X7R-dependent mechanism of action, which may explain why AZD9056 failed in cohorts with background anti-inflammatory agents. The use of AZD9056 for those with RA was abandoned as a potential therapeutic strategy.

Crohn's Disease (CD)

The clinical efficacy of AZD9056 was repurposed for investigation in phase II clinical trials in a population with CD on a similar background of anti-inflammatories as the RA cohort. (241) While cytokine release is a crucial driver in CD, with neutralisation of their activity shown to be effective in animal models of intestinal inflammation, the purpose of this trial was to evaluate the impact of AZD9056 on pain as opposed to clinical remission of CD. (242,243) In participants allocated to receive 200 mg of AZD9056 for 28 days, significant improvements in measures associated with chronic pain and mental health were noted. (241) Improvements in chronic pain were observed without effecting plasma CRP concentration. However, the influence of ATP-induced IL-1 β release from ex vivo-derived monocytes in this population was not evaluated. (241) While the failure of

AZD9056 to resolve CRP concentration in this cohort may be dependent on P2X7R limited relevance in CD, it could also be explained by the relatively short treatment duration. Alternatively, CRP as a surrogate marker is also limited due to its lack of specificity. Compared to the RA AZD9056 trial, the treatment duration was limited to 28 days, where the dose received was half of what was tested in the initial trial. (241) Despite not being included as a primary endpoint, the proportion of patients that entered clinical remission, while by number was higher in the AZD9056 cohort, did not satisfy statistical thresholds from participants allocated to placebo. (241) Under these circumstances, AZD9056 was not pursued as a potential treatment for chronic pain in CD due to its modest benefits. (241)

Aftermath and insights from ‘classical’ inflammatory trials

In the initial AZD9056 trial involving a RA cohort, Stock et al. suggested that P2X7R antagonism alone might be insufficient to achieve clinical remission, due to the complexity of immune-mediated disorders like RA and CD. (244) The current understanding of the mechanisms underlying these disorders remains incomplete, though it is recognised that patients benefit from broad-spectrum anti-inflammatories. The disadvantage of these approved immunomodulatory compounds is their suppression of the immune system, which is associated with higher incidences of infections. (13,60) Moreover, for the proportion of patients that do not achieve clinical remission despite treatment with common immunosuppressants, therapy with a P2X7R antagonist promised further pharmacotherapeutic intervention. However, considering the unsuccessful outcome of AZD9056 in clinical trials against a backdrop of other clinically proven anti-inflammatory agents, its potential application in conditions lacking approved anti-inflammatory treatments warrants consideration. From a CVD perspective, the substantial clinical benefits of inhibiting single molecules such as IL-1 β and IL-6 on future CVD events suggest a potential application for AZD9056 in atherosclerosis. Targeting these cytokines through the P2X7R offers several advantages over traditional immunosuppression. The P2X7R modulates the processing and release of IL-1 β and IL-18, two cytokines that promote IL-6 and TNF- α release. P2X7R antagonism should suppress several key cytokines through this mechanism, potentially improving the clinical benefit. Furthermore, considering that the activation of the receptor depends on sterile inflammatory conditions, a P2X7R antagonist will have minimal immunosuppressive effects. Under these circumstances, the use of AZD9056 warrants investigation for the treatment of CAD or other CVD conditions with inflammatory involvement. Since then, the patent for AZ adamantane derivatives, which covered AZD9056, has expired. AZD9056 was not evaluated further in RA cohorts due to the failed phase II trial in patients with a background of anti-inflammatories. AZ disclosed two patents that covered the synthesis of a novel class of quinoline derivatives identified as part of the panel in 2003. (245) The US patents for these compounds have since been extended until 2030 and cover one novel P2X7R antagonist, LDD-2598. (246) While interest in P2X7R antagonists has somewhat dissipated since these trials, the significance of P2X7R in the pathogenesis of common degenerative and life-threatening conditions has resulted in the persistent investment of pharmaceutical companies and research groups in the development of novel classes of P2X7R antagonists.

Table 3. Common splice variants identified on the P2X7R. Outlined is the protein variation, effect to the protein structure and function.

Isoform	Protein variation	Effect	Function	Citation
P2X7A	Full-length receptor		Normal function. Expressed across tissues and PBMCs.	146, 204
P2X7B	Stop codon between exons 10 and 11	Deletion of last 249 amino acids. Replaced by 18 different amino acids.	Function channels but cannot form pores. Co-expression with a P2X7RA increases calcium mobilisation and pore formation. Isoform is more abundantly expressed across tissues. Expressed in PBMCs.	146, 204
P2X7C	Deletion of exon 4. Stop codon between exons 10 and 11	Modification to part of the ectodomain. Deletion of last 249 amino acids. Replaced by 18 different amino acids.	Not characterised.	
P2X7D	Deletion of exon 5.	Modification to part of the ectodomain.	Not characterised.	
P2X7E	Deletion of exon 7 and 8. Stop codon between exons 10 and 11	Modification to part of the ectodomain. Deletion of last 249 amino acids. Replaced by 18 different amino acids.	Not characterised.	
P2X7F	Deletion of exon 4 and 8.	Modification to the ectodomain.	Not characterised.	
P2X7G	Insertion of the N3 exon between exons 2 and 3. Stop codon between exons 10 and 11.	Introduction of the start codon and deletion of TMD1. Deletion of the last 249 amino acids. Replaced by 18 different amino acids.	Not characterised.	
P2X7H	Insertion of the N3 exon between exons 2 and 3.	Introduction of the start codon and deletion of TMD1.	No pore-formation or calcium mobilisation upon BzATP stimulation. Low expression across tissues and PBMCs. Co-expression with P2X7R results in non-function heterotrimers.	146
P2X7I	Frameshift mutation on exon 8 produces a stop codon with one-third of the extracellular loop.	Short receptor composed of 258 amino acids. No C-terminus domain, TMD2, or one-third of the ectodomain.	Non-functional receptor.	207
mP2X7K	Addition of exon in intron between exons 1 and 2.	Modification to N-terminus and TMD1.	Increases activity in response to ATP. Exclusively expressed in T cells in rodent.	208

sp	Q99572	P2RX7_HUMAN	MPACSCSDVQYETNKVTRIQSMNYGTIKWFFHVIIFSVCYCFALVSDKLYQRKEPVISS	60
sp	Q64663	P2RX7_RAT	MPACCSWNDVQYETNKVTRIQSVNYGTIKWLHMTVFSYVSFALMSDKLYQRKEPLISS	60
sp	Q9Z1M0	P2RX7_MOUSE	MPACCSWNDVLQYETNKVTRIQSTNYGTIKWLVHMIVFSYISFALVSDKLYQRKEPVISS *****.*****.*****.*****.*****.*****.*****.*****.*****.*****	60
sp	Q99572	P2RX7_HUMAN	VHTKVKGIAEVKEEIVENGVKLVHSVFDTADYTFPLQGNSEFFVMTNFKLKEGQEQRLCP	120
sp	Q64663	P2RX7_RAT	VHTKVKGVAEVTENVTEGGVTKLVHGIFDTADYTLPLQGNSEFFVMTNFKLKEGQEQRLCP	120
sp	Q9Z1M0	P2RX7_MOUSE	VHTKVKGIAEVTENVTEGGVTKLVHGSIFDTADYTFPLQGNSEFFVMTNFKLKEGQEQRLCP *****.*****.*****.*****.*****.*****.*****.*****.*****.*****	120
sp	Q99572	P2RX7_HUMAN	EYPTRTTLCSDDRGCKKGWMDPQSKGIQTGRGVVYEGNQKTCVSAWCPIEAVEEAPRPA	180
sp	Q64663	P2RX7_RAT	EYPSRGKQCHSDQGCIGWMDPQSKGIQTGRGCIYPDQKRKKTCEIFAWCPAEEGKEAPRPA	180
sp	Q9Z1M0	P2RX7_MOUSE	EYPRRGAQCSSDRRCKKGWMDPQSKGIQTGRGVYDTRKTKCEVSAWCPTEEEKEAPRPA *** *	180
sp	Q99572	P2RX7_HUMAN	LLNSAENFTVLKNNIDFPGHNYTTRNILPGLNICTFHKTKNPQCPIFRLGDIFRETGD	240
sp	Q64663	P2RX7_RAT	LLRSAENFTVLKNNIDFPGHNYTTRNILPGLNICTFHKTKNPQCPIFRLGDIFQETGE	240
sp	Q9Z1M0	P2RX7_MOUSE	LLRSAENFTVLKNNIHFPGHNYTTRNILPTMNGSCITFHKTKWDPQCSIFRLGDIFQEAGE **..*****.*****.*****.*****.*****.*****.*****.*****.*****.*****	240
sp	Q99572	P2RX7_HUMAN	NFSDVAIQGGIMGIEIYWDNCNLDWFWHHCRCPKYSFRLDDKTNNVSLPGYNFRIYAKYYK	300
sp	Q64663	P2RX7_RAT	NFTEVAVQGGIMGIEIYWDNCNLDWSHRCRCPKYSFRLDDKTYNLSLPGYNFRIYAKYYK	300
sp	Q9Z1M0	P2RX7_MOUSE	NFTEVAVQGGIMGIEIYWDNCNLDWSHHCRCPRYSFRLDDKNTDESFPVPGYNFRIYAKYYK **.:**.:*****.*****.*****.*****.*****.*****.*****.*****.*****.*****	300
sp	Q99572	P2RX7_HUMAN	ENNVEKRTLKIVFGIRFDILVFGTGKGKFDIIQLVVYIGSTLSYFGLAAVDFIDLINTYSS	360
sp	Q64663	P2RX7_RAT	ENNGEKRTLKAFGVIRFDILVFGTGKGKFDIIQLVVYIGSTLSYFGLATVCIDLINTYAS	360
sp	Q9Z1M0	P2RX7_MOUSE	ENNVEKRTLKAFGIRFDILVFGTGKGKFDIIQLVVYIGSTLSYFGLATVCIDLINTYSS **.:*****.*****.*****.*****.*****.*****.*****.*****.*****.*****	360
sp	Q99572	P2RX7_HUMAN	NCCRSHIYPWCKCCQPCVVNEYYYRKKCESIVEPKPTLKYSVSVFDESHIRMVNQQLLGRS	420
sp	Q64663	P2RX7_RAT	TCCSRSVYPSCKCCEPCAVNEYYYRKKCEPIMEPKPTLKYSVSVFDEPHIMVMDQQLLGRS	420
sp	Q9Z1M0	P2RX7_MOUSE	AFCSRGVYPCCKCCEPCVNEYYYRKKCESIMEPKPTLKYSVSVFDEPHIMVMDQQLLGRS *** :** *****.*****.*****.*****.*****.*****.*****.*****.*****.*****	420
sp	Q99572	P2RX7_HUMAN	LQDVKGQEVPRPAMDFTDLRLPLALHDTPIPGQPEEIQLLRKEATPRSRDSPVWCQCG	480
sp	Q64663	P2RX7_RAT	LQDVKGQEVPRPQTDPLRLPLALHDTPIPGQPEEMQLLQIEAVPRSRDSPVWCQCG	480
sp	Q9Z1M0	P2RX7_MOUSE	LQVVGKQEVPRPQMDFTDLRLPLALHDTPLTPGQSEIQLLHEEVAPKSGDSPVWCQCG ** *****.*****.*****.*****.*****.*****.*****.*****.*****.*****	480
sp	Q99572	P2RX7_HUMAN	SLCPSQLPESHRCLEELCCRKKPGACITTSFLFRKLVLRSRHLVQLLQLLYQEPLALDVS	540
sp	Q64663	P2RX7_RAT	NCLPSQLPENRRALEELCCRKKPGQCITTSFLFSKIVLSREALQLLQLLYQEPLALEGEA	540
sp	Q9Z1M0	P2RX7_MOUSE	NCLPSRLPEQRRALEELCCRKKPGRCITTSKLFHKLVLRSRTLQLLQLLYQDPLVLGEEA ..****.***.:*.*****.*****.*****.*****.*****.*****.*****.*****.*****	540
sp	Q99572	P2RX7_HUMAN	TNSRLRHCAIRCYATWRFSGQDMADFAILPSCCRWRIRKEFPKTEGQSYSGFKSPY	595
sp	Q64663	P2RX7_RAT	INSKLRHCAYRSYATWRFVSGQDMADFAILPSCCRWRIRKEFPKTEGQSYSGFKSPY	595
sp	Q9Z1M0	P2RX7_MOUSE	TNSRLRHRAIRCYATWRFSGQDMADFAILPSCCRWRIRKEFPKTEGQSYSGFKSPY *****.*****.*****.*****.*****.*****.*****.*****.*****.*****	595

	Human	Rat	Mouse
1: sp Q99572 P2RX7_Human	100	80.34	80.84
2: sp Q64663 P2RX7_Rat	80.34	100	85.04
3:sp Q9ZIM0 P2RX7_Mouse	80.84	85.04	100

RA

46

shape of the binding domain between human and rodent receptors. (144) While preclinical studies for CE-224,535 were not reported, (203) the compound was determined to have plasma acceptable clearance in pharmacokinetic studies completed in rats, dogs, and monkeys (plasma clearance: 11, 12, and 16, respectively) and bioavailability evaluated in dogs and monkeys (bioavailability % 59 and 22, respectively). (247) Its initial aim as a CNS penetrant separated it from previously developed P2X7R antagonists and opened new avenues in targeting CNS disorders, a common challenge of small molecules.

Much like AZD9056, CE-224,535 was targeted for a population with recurrent RA with inadequate remission despite receiving ongoing MTX treatment (244) In this cohort, trough blood concentrations of CE-224,535 was estimated to exceed those required for IL-1 β release inhibition, which was derived from whole-blood stimulation assay. (244) Despite this, CE-224,535 failed to demonstrate efficacy in phase II trials, with no improvement in ARC20 response in a 12-week study. (244,247) Since the concentrations reached in this trial are appropriate to achieve adequate IL-1 β inhibition, validated by IL-1 β release from ex vivo-derived monocytes, this confirms the poor efficacy of P2X7R in RA. Additionally, like the AZD9056 trial, CE-224,535 aimed to reduce unresolved inflammation in combination with other anti-inflammatories. Limitations in this strategy were demonstrated in a murine MI model, where Anakinra was treated in combination with a P2X7R antagonist. (229) Briefly, treatment with Anakinra combined with P2X7R antagonist A740003 did not further improve measures of heart function compared to either A740003 or Anakinra alone. (229) These anti-inflammatories also target adenosine G-protein-coupled receptors, adenosine A2A and adenosine A2B, (249,250) that diminish the cytosolic accumulation of proinflammatory cytokines through NF- κ B. Under these circumstances, therapeutic inhibition of the P2X7R may be useless in combination with other anti-inflammatories. Recent studies have highlighted alternative strategies for the treatment of RA, such as the use of Chimeric Antigen Receptor T cell therapies. (251)

Osteoarthritis

Much like the trajectory of the AZ compound, a second clinical trial was established to evaluate the use of CE-224,535 as an analgesic in a cohort with osteoarthritis. (252) The results of this study are constrained to a clinical trials report where CE-224,535 was found to be ineffective compared to placebo, leading to the termination of the study. (252) Comparatively to other tested P2X7R antagonist, CE-224,535 was determined to be safe and well-tolerated in participants at concentrations up to 1.6 g per day for two weeks. (253) No adverse events were reported, with side effects limited to increased gastrointestinal symptoms. After the termination of the trial, Pfizer has never publicly investigated a P2X7R antagonist for the treatment of inflammatory-driven disease states. Instead, other large pharmaceutical companies have dominated the space, with several successful developments based on novel structures.

GlaxoSmithKline (GSK)

Neuropathic pain

A previous limitation of the other compounds was their loss of sensitivity at the rodent P2X7R, preventing extensive assessment of the exact compound in relevant preclinical models. GSK disclosed four consecutive patents between 2008 and 2011, which outlined the preparation and use of CNS-permeable P2X7R antagonists for treating inflammatory conditions. (247,254–258) Screening of a compound library led to the identification of GSK-1482160, which was included on a 2008 patent as part of a series of pyroglutamic acid amides. (209,212) As mentioned above, GSK-1482160 demonstrates potency across several species, including rodent and human receptors, with a half-maximal inhibitory concentration (IC₅₀) value of 316 nM and 3.16 nM, respectively. (254,259) Based on the compound dynamics, GSK-1482160 was proposed to be a negative allosteric modulator at the P2X7R, (254) a pharmacological characteristic suggested to be beneficial to achieve adequate inhibition. Allosteric antagonism has certain advantages over traditional orthostatic inhibition, specifically pertaining to the selectivity at the allosteric site. (260) From the context of P2X7R structural relationships, evidence suggests that allosteric modulators prevent a conformational change to the receptor that prevents expansion of the lower body domain –referred to as the macropore. (250) Moreover, GSK-1482160 demonstrated appropriate PK properties for clinical development with a half-life of 1.5 hours. (259)

The development of antagonists that retained potency at both human and rodent P2X7Rs was significant in the evolution of P2X7R antagonists. GSK-1482160 was the first to display therapeutic potential in preclinical studies. Particularly, the compound reduces nerve-induced allodynia, compared to gabapentin, a gold standard analgesic in a rodent model of chronic constriction injury. (254,259) GSK-1482160 was efficacious in inflammation-induced pain associated with arthritic hypersensitivity in a rodent model. (254,259) Consistent with previously published literature, P2X7R^{-/-} mice were protected against inflammatory-induced neuropathic pain. (261) These results contributed to the progression of GSK-1482160 into phase I clinical trials. (254) However, the trials were terminated before the completion of the study due to concerns over the doses required for sustained treatment. (254) In healthy participants, adequate plasma concentrations of GSK-1482160 could not be reached within safety margins to elicit a clinical benefit. (254) Based on initial modelling, compounds that targeted P2X7R-induced IL-1 β release must reach concentrations required to achieve a 90% reduction in IL-1 β to achieve efficacy. (254) This feature was attributable to both AZD9056 and CE-224,535, which have previously been described. On these grounds, further evaluations for the use of GSK-1482160 as an analgesic were terminated.

Positron emission tomography (PET) tracer

More recently, the P2X7R has been proposed as a biomarker to detect early inflammation using PET imaging. (262) Due to the abundance of P2X7R in resident microglia in the CNS, which have shown to be critical in inflammatory signalling, phagocytosis (263), and the clearance of apoptotic cellular material, PET tracers against the P2X7R have been suggested for the detection of neuroinflammation. (262,264) In the context of atherosclerosis, the utility of a radiolabelled P2X7R antagonist may help detect vascular inflammation before

the development of atherosclerosis. The relatively lower plasma concentrations reached with GSK-1482160 made it an appropriate candidate for conversion into a PET tracer. After administration, [^{11}C]GSK-1482160 was shown to cause an immediate increase in tracer levels in mice treated with LPS 72 hours before. (265) The tracer stabilised in mouse tissue within 10 minutes in peripheral tissue. (266) However, [^{11}C]GSK-1482160 was not evaluated further for peripheral indications. In an LPS-induced neuroinflammation mode, brain activity increased after [^{11}C]GSK-1482160 detection. (265) Treatment with GSK-1482160 reduced brain activity and confirmed that [^{11}C]GSK-1482160 was selective for P2X7R-dependent neuroinflammation. (265) These results were replicated in rats, where [^{11}C] GSK-1482160 was associated with encephalomyelitis in the spinal cord. (267) In this study, [^{11}C]GSK-1482160 demonstrated adequate penetrance and tissue distribution. (267) Based on these studies, [^{11}C]GSK-1482160 was proposed as a potential tracer for detecting neuroinflammation in patients with multiple sclerosis.

For its use as a diagnostic tool, [^{11}C]GSK-1482160 was investigated in a phase I clinical trial in healthy participants. (266) Its detection was dominated in the liver and other peripheral organs, (266) making it appropriate to detect peripheral inflammation. Brain uptake of [^{11}C]GSK-1482160 was estimated to be limited to 2% of the injected dose and, thus, was insufficient to reach concentrations required to detect neuroinflammation. (266) The requirements for high concentrations of radiation would make [^{11}C]GSK-1482160 inappropriate for use as a diagnostic tool in neuroinflammation. Given the initial interest as a brain tracer, further evaluations of this compound were terminated. There is a potential missed opportunity for using GSK-1482160 as a PET ligand, where repurposing should be considered to diagnose early CVD. Since then, further structural analogues of GSK-1482160 have been developed to improve the half-life and brain penetrance of the compound. (268) Notably, the compound has been subjected to halogen substitutions of either F-, Br- or I- to the benzyl group. (268) Modest improvements in potency have been reported. However, since 2019, there have been no further publications on GSK-1482160 and its structural variants, although it is understood that preclinical evaluations were to be conducted with [^{11}C]Br-GSK-1482160 and [^{11}C]I-GSK-1482160. As previously highlighted, shifting focus to CVD might yield more promising outcomes for using P2X7R antagonists as a radiotracer, particularly given that the requirement for blood-brain penetration is eliminated.

Table 4. Clinical evaluation of P2X7R antagonist in human trials.

Clinical Development	Phase	Cohort	Main Outcome(s)	Safety/Adverse Events	Citations
AstraZeneca					
AZD9056	II	RA, IBD, COPD	No therapeutic efficacy in RA. No efficacy in CD or COPD. Further trials were terminated.	Safe and well-tolerated up to 400 mg daily for 24-weeks. Dose-dependent gastrointestinal events.	231, 240
Pfizer					
CE-224,535	II	RA, OA	No efficacy in RA. Failed to demonstrate efficacy as an analgesic for osteoarthritis. Further trials were terminated.	Safe and well-tolerated up to 1600 mg daily oral administration for 12-weeks. Gastrointestinal symptoms most often reported.	243
GlaxoSmithKline					
GSK-1482160	I	Pain	Failed to reach required therapeutic plasma concentrations within safety margins in phase I clinical trials. Discovered to be unsuitable as a radiotracer for neuroinflammatory indications.	1 event of accelerated idioventricular rhythm and hospitalisation on 1000 mg. Symptoms resolved without interference.	253, 266
Janssen					
JNJ-54175446	II	MDD	Demonstrated mood modulatory effects in healthy participants treated with dexamphetamine. Mood modulatory effects in phase II trials in patients with MDD. Reduction in IL-1 β after BzATP-stimulation.	Safe and well-tolerated in healthy participants. The most common complications reported were headache (18.6%) and back pain and fatigue (8.5%). Adverse events were reported after treatment with dexamphetamine with symptoms resolved in 24-hours.	271, 273, 274
JNJ-55308942	II	MDD	Currently under investigation for bipolar depression in phase II clinical trials.	No published clinical data – however, suspected safety and tolerability in participants as compound progressed into phase II clinical trials. Acceptable cardiovascular profile and plasma concentrations in preclinical studies.	275
Evotec					
EVT-401	I	RA	Reported to have progressed to phase II clinical trials. No publication of data.	Reported to be safe and well-tolerated in released public announcements. No publication of clinical data.	287, 288

Janssen

JNJ-54175446

From 2009 to 2016, Janssen disclosed six patents covering the preparation and use of P2X7R antagonists for the treatment and diagnosis of neuropsychiatric and neurodegenerative conditions (216,269), of which only two will be covered in this literature review. (270,271) Within a similar timeframe compound development to GSK, Janssen succeeded in developing novel P2X7R antagonist scaffolds that penetrate the blood brain barrier and retain interspecies potency. (216) Several compounds were identified from these patents as suitable for clinical development and were recently evaluated in clinical trials for treating neuropsychiatric diseases. JNJ-54175446 was recognised as a lead candidate from a series of novel 4-(R)-methyl-6,7-dihydro-4H-triazolo [4,5-c] pyridines outlined in a 2014 patent. (271) It was determined to be selective and potent across several species. (271) In vivo, pharmacokinetics showed that JNJ-54175446 exhibited adequate pharmacokinetic properties, pertaining to its longer half-life than previous compounds, plasma bioavailability and favourable safety profile. (271,272) Janssen published no further preclinical animal studies concerning the efficacy of JNJ-54175446.

In healthy participants, JNJ-54175446 was evaluated for its safety in phase I clinical trials. (273) Concentrations up to 600 mg were safe and well-tolerated, with no adverse events reported throughout the trial. (273) A subsequent trial in healthy participants validated these results in a separate cohort of healthy participants. (272) Participants were also evaluated for the potential of JNJ-54175446 to improve measures of mood such as alertness, calmness, and satisfaction. (273) Compared to the placebo, those allocated to JNJ-54175446 did not demonstrate substantial differences in mood, (273) although this result is not entirely unexpected. In the absence of a pre-existing inflammatory risk, treatment with a P2X7R should hold no benefit or disadvantage in the absence of a stimulus and, thus, is consistent with the literature. To confirm this mechanism, JNJ-54175446 was evaluated in healthy males under dexamphetamine stress, which has been shown to induce neuroinflammation. (272) JNJ-54175446 was demonstrated to diminish stress-induced behaviour such as finger-tapping and saccadic inaccuracy and improved measures of calmness and satisfaction. (273) Changes in behaviour were associated with sustained suppression of IL-1 β in whole-blood stimulation assay. (260) Due to the success of these early phase I clinical trials, JNJ-54175446 progressed into phase II trials in a cohort with major depressive disorder. Comparatively to prior trials, patients in this phase II study were predominately treatment-free, although a small population had a background of selective serotonin reuptake inhibitors. (274) In this study, it was unclear whether JNJ-54175446 was effective in treating major depressive disorder as the behaviour of total sleep deprivation to induce anti-depressant effects differed from previously published literature. (274) Despite this, scales of anhedonia improved after treatment with JNJ-54175446, suggesting mild improvements in mood associated with major depressive disorder. (275) Like other trials, this was associated with a substantial reduction in IL-1 β levels from whole-blood LPS stimulated assay. (274) This was particularly notable in those who started treatment before exposure to total sleep deprivation. (274) JNJ-54175446 represents an initial success of P2X7R antagonists in clinical trials and reignites optimism for treating conditions with less conventional inflammatory origins, such as atherosclerosis.

JNJ-55308942

As an alternative to JNJ-54175446, Janssen also identified JNJ-55308942 for clinical development from a series of fused triazole pyridines. (270,276) JNJ-55308942 also demonstrate adequate selectivity for P2X7R across several species. (270,276) Comparatively to others in the series, JNJ-55308942 demonstrated improved compounds solubility, resulting in a modest reduction in half-life. (270) Like the previously generated P2X7R antagonists, JNJ-55308942 was developed for neuroinflammatory indications. JNJ-55308942 was evaluated in a rat model of stressed-induced depression, where rats were demonstrated to be protected against anhedonia. (276,277) Treatment with JNJ-55308942 was as effective as Imipramine (276)— a common tricyclic antidepressant against inflammation-induced mood destabilisation. (278) This was further validated in a Bacillus Calmette-Guerin-induced neuroinflammation model in mice, where JNJ-55308942 treated mice consumed higher quantities of sucrose than vehicle-treated mice. (276) Based on these results and the success of JNJ-54175446, JNJ-55308942 was evaluated in phase I trials, although the results of these findings were not published. (216) No further announcements have been made about the clinical development of this compound.

JNJ-54173717

Lastly, Janssen disclosed JNJ-54173717 from a series of [1,2,4] triazolo[4,3-A]pyrazines patented in 2014. (279) Drug screening revealed that JNJ-54173717 was potent, particularly to human and rodent receptors. (280,281) Like other antagonists, JNJ-54173717 was also indicated for neuroinflammatory disorders and was evaluated in vivo to have adequate in vivo receptor occupancy in the rat brain. (280) Due to its substantial bioavailability in the brain and plasma after oral administration, JNJ-54173717 was suggested to be a suitable candidate as a PET ligand. (280,282,283) Like GSK-1482160, JNJ-54173717 was ¹¹C-labeled and found to bind with high affinity within the white matter of the mouse brain. (281) This response was diminished in P2X7R^{-/-} mice, indicating that the compound was selective for the P2X7R. Further evaluation in the rhesus monkey brain demonstrated that [¹¹C]JNJ-54173717 was selectively bound to the P2X7R, and the distribution of the radioligand was homogenous throughout the brain. (282) [¹¹C]JNJ-54173717 progressed to clinical trials as a PET ligand in participants with Parkinson's Disease. (282) It exhibited a stable metabolism compared to previous P2X7R PET tracers, which resulted in a more appropriate acquisition time. (282) Despite this, [¹¹C]JNJ-54173717 could not detect differences in radiotracer uptake between Parkinson's Disease and healthy controls, (282) which may indicate a lack of specificity of P2X7R for detecting Parkinson's Disease. Consequently, no further studies were carried out with JNJ-54173717, and since then, no other compounds have been modified for use as PET tracers.

Evotec

EVT401 was disclosed as part of 2007 Evotec patent of bicycloheteroaryl P2X7R antagonists an IC₅₀ of 9.3 nM and a half-life of 0.93 hours in human liver microsomes. (284,285) The structure of EVT401 featured a unique isoquinolinone carboxamide core, and a later patent revealed many compounds of the same scaffold substituted with an adamantane motif, a characteristic of AZD9056 reported in 2003. (286) Evotec retained this adamantane cage in later disclosures on different heterocyclic cores, although no analogues, except EVT401, were progressed

into clinical evaluation. (286,287) At the time of writing, information about the progression of EVT401 is limited to vague announcements made through the public webpage of Evotec Pharmaceutical, (288) with no further data published related to its scientific assessment. Though patented in 2007, Evotec only disclosed this lead when Zhu et al., 2017 published an article describing its characterisation. (285) EVT401 was reported to have entered phase I clinical trials in 2009 with the support of Conba Pharmaceuticals and was found to be safe and well-tolerated in male participants with no underlying conditions. (288) The compound also blocked IL-1 β release in patient-derived whole blood stimulated with BzATP at a concentration well within safety margins. However, the data was not disclosed, and no further specifications were reported. (288,289) Evotec granted Conba Pharmaceutical exclusive rights to the development and commercialisation of EVT401 in China after the success of phase I clinical trials. (289) EVT401 was reported to have entered phase II clinical trials to be evaluated in a cohort with unresolved, persistent RA. However, nothing further has been released since the end of the trial in 2009.

Future development of P2X7 receptor antagonists

A future niche in cardiovascular disease

There is a clear unmet clinical need for effective pharmacotherapeutics that reduce inflammation contributing to CVD. The P2X7R is a critical therapeutic strategy for treating chronic inflammatory conditions such as CAD and HFpEF. While many of the aforementioned P2X7R antagonists failed to demonstrate efficacy against crucial clinical endpoints, their effectiveness was evaluated in situations with a strong background of immunotherapies. Under these circumstances, pathways modulated by P2X7R are subdued by traditional immunosuppressants, rendering a P2X7R antagonist useless in combination with these treatments. In participants with major depressive disorder, success in trial endpoints was achieved with JNJ-54175446, although the effectiveness of the sleep deprivation challenge underwhelmed the results. (274) However, a benefit of P2X7R remains for the treatment of CVD due to its crucial role in the modulation of IL-1 β and IL-18 production and release. (141,164,182) A significant driver of IL-6 synthesis is the expression of active IL-1 β , where IL-6 acts as a secondary messenger cytokine in inflammation. (20) From a pharmacological perspective, suppression of inflammation through P2X7R holds tremendous potential for antibody therapies since inhibition is extended to several inflammatory cytokines instead of single molecules. The successful inhibition of IL-1 β and IL-18 release from BzATP-induced monocytes from patients with CAD supports the value of examining these agents within this vital cohort. Moreover, as P2X7R activation is strictly regulated through extracellular ATP accumulation from injured cells, reducing off-target effects compared to current immunosuppressants such as Canakinumab and Colchicine remains a distinct benefit of P2X7R therapies. Thus, while pharmaceutical companies have become complacent to P2X7R as a therapeutic target, clinical evaluation in a cohort with CAD may reverse the class's fortunes. Like the abovementioned trials, immunomodulation through P2X7R should effectively reduce first-time and recurrent CVD events.

The development of P2X7R antagonists for the treatment of CVD is more straightforward than what has been accomplished in P2X7R drug discovery thus far. Notably, the development of small molecules that cross the blood brain barrier was a challenge in P2X7R drug development that was overcome early on. While blood brain barrier permeability is essential for diagnostic and therapeutic value in neuroinflammatory conditions, it is inconsequential for treating CVD. Given these circumstances, the ideal physicochemical properties of a P2X7R antagonist can be less stringent for compounds that remain peripherally restricted. Parameters for lipophilicity and molecular weight for these compounds can afford to be broader, allowing for greater flexibility in compound design and optimisation. Moreover, concentrations required to achieve adequate plasma concentrations required for effective IL-1 β modulation can be lower, as was demonstrated in PET studies, where peripheral uptake was substantial compared to CNS detection. (282,290) Thus, compounds aimed at peripheral disease states can afford to be slightly less potent, given their broader capability for plasma bioavailability.

A substantial limitation in the design of P2X7R antagonists would relate to its preference for allosteric or orthosteric inhibition. A competitive antagonist with a preference for the orthosteric site must outcompete ATP to achieve adequate P2X7R modulation. For this type of binding to be successfully attained, the affinity and potency of the compound must outperform endogenous ligands. Comparatively, a negative allosteric modulator may convey certain advantages, as the binding site is juxtaposed to the ATP site, (151) removing the requirement for competitive inhibition. However, there are substantial limitations to the design of negative allosteric modulator P2X7R antagonists. Namely, structural studies indicated that the allosteric pocket of the P2X7R becomes concealed by a conformational change to part of the ectodomain following ATP binding. Given that a P2X7R antagonist would not be received before the onset of the disease, this could pose considerable issues in effectiveness. A more appropriate approach might involve the design of P2X7R antagonists with a promiscuous binding pattern, facilitating their activity at allosteric and orthosteric sites. Under these circumstances, promiscuous antagonists can compete with ATP at lower risk and better occupy allosteric sites.

Development of an adamantane benzamide P2X7R antagonist to target inflammation in CVD

Having established the essential criteria which must be met for an effective P2X7R antagonist, our team proceeded with the development of a series of adamantane benzamide P2X7R antagonists. (291) The first-generation compound was designed around the adamantane amide structure originally proposed in 2003 by AZ. (237) This initial structure exhibited heightened potency at the human P2X7R while exhibiting substantial metabolic liability. (237,291) Thus, despite its promising activity, its half-life was reduced to minutes, necessitating further optimisation for improved pharmacokinetic properties. The simplicity of the initial structure indicated that the metabolic liability originated from the adamantane head, which was suggested to exhibit a potential weakness prone to degradation. To address this, our team implemented a series of strategic substitutions to firstly confirm this hypothesis and then to enhance the metabolic stability of the compound. (262,291) This approach led to the selection and successful development of PKT100, which exhibited a fluorine-for-hydrogen substitution of the adamantane cage. PKT100 was demonstrated to have improved

pharmacokinetic properties, without substantial effects on potency compared to other P2X7R antagonists. (291) Additionally, while many P2X7R antagonists at the time faced significant interspecies variability that hindered preclinical evaluation, PKT100 demonstrated effectiveness across both human and mouse receptors. (291) Although its half-life was shorter in mice than in humans, its overall pharmacokinetic profile was an advancement.

Early P2X7R antagonists designed to penetrate the blood brain barrier often faced poor penetration due to suboptimal molecular weight, lipophilicity, and electrostatic properties. (269) While this is less of an issue for compounds targeting peripheral tissues, the initial design of PKT100 was specifically aimed at neuroinflammatory disease-states, (291,292) as reflected by the pharmacokinetic properties. However, the potential utility of PKT100 in CVD was realised during pharmacokinetic studies which revealed favourable biodistribution of [¹¹C]PKT100 in peripheral organs following intravenous administration in rats. (262) This study showed efficient uptake of PKT100 in the liver, heart and kidney of rats. (262) Given this evidence, our team repurposed PKT100 as a potential therapeutic for right ventricular HF associated with pulmonary hypertension. (293) Currently, there are no approved pharmacotherapies for the treatment of right ventricular HF, and thus diagnosis is often associated with a reduced life quality and expectancy. (294,295) In a mouse model of bleomycin induced pulmonary hypertension, which is associated with total organ fibrosis, PKT100 demonstrated protection against RV HF and mortality compared to vehicle treated mice. (293) Notably, the cardioprotective effects of PKT100 were found to be independent of a reduction in pulmonary hypertension itself – which has numerous pharmacotherapeutic options and indicates a specific biological effect on the right ventricle (293) Moreover, in a more recent study, our team also evaluated the potential of PKT100 to reduce heightened IL-1 β release from PBMC-derived monocytes co-stimulated with BzATP in a cohort with CAD. (221) PKT100 was shown to reduce BzATP-induced pro-inflammatory cytokine release in these patients, (221) which indicates a potential role for the P2X7R in the inflammatory process associated with CAD and highlights the broader therapeutic potential of PKT100 in CVD.

Final insights and remarks

The emergence of inflammation as a significant contributor to ASCVD and related metabolic conditions is now recognised. However, we are only “scratching the surface” when measuring or targeting it therapeutically. There is an unmet need for improved blood-based biomarkers of dysregulated inflammation driving atherosclerotic activity to enhance the precision of CAD treatment. The Food and Drug Administration’s FDA approval of Colchicine for treating ASCVD in patients with adequate renal and hepatic function marks a new milestone in the development of immunomodulatory strategies for the treatment of ASCVD. While its use is associated with improved clinical outcomes, its limitation to patients with adequate hepatic and renal function restricts its use in older populations that would benefit from immunomodulatory strategies. Particularly, since the identification of clonal hematopoietic mutations, resulting in heightened inflammatory burden and worst prognosis after MI, (296) safe pharmacotherapeutic interventions are warranted for the treatment of ASCVD. Given the substantial

role of IL-1 β in ASCVD, we have identified the P2X7R as a unique strategy to modulate the inflammatory contribution in CVD. The receptor is recognised for regulating the production and release of mature IL-1 β and IL-18, transitioning them from their inactive precursor form in response to ATP-induced tissue injury. Downregulation of these cytokines has a broader impact on downstream mediators, including IL-6 and CRP, where IL-6 has demonstrated predictive value in ASCVD and HF. Thus, targeting the P2X7R expands the therapeutic utility and distinguishes it from traditional anti-inflammatory treatments for CVD.

Over the last two decades, efforts in P2X7R drug discovery have identified several classes of P2X7R antagonists that have demonstrated properties suitable for clinical development. However, successful translation of these compounds in the setting of inflammatory diseases has been hindered by unsuccessful clinical trials that have required P2X7R antagonists to perform against a background of other immunosuppressants. This challenge was exemplified in a preclinical study, where treatment with Anakinra in combination with a P2X7R antagonist failed to achieve a synergistic reduction in inflammation. Remarkably, treatment with a P2X7R antagonist alone exhibited anti-inflammatory benefits comparable to those observed with dual therapy in a murine model of MI. The failure of this novel class of immunomodulatory compounds has resulted in a decline in engagement and investment from larger pharmaceutical companies in the discovery of drugs targeting the P2X7R. However, given the substantial influence of inflammatory pathways in CVD, we propose that CVD represents an ideal opportunity to reignite interest in drug discovery efforts targeting inflammation, considering the sparseness of ineffective management strategies for CAD. Moreover, synthesising radiolabelled P2X7R-targeting compounds may also offer a potential diagnostic opportunity in detecting early vascular inflammation in atherosclerosis and related metabolic syndromes. This work will address these limitations and explore the potential of a novel series of P2X7R antagonists for treating the inflammatory contribution to CVD.

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Hypotheses and Aims

Chapter 3

Hypothesis: Targeting the P2X7R could be an effective therapeutic strategy against inflammation in CAD

Aim 1: To assess P2X7R expression across PBMCs from patients with acute MI and CAD using high-resolution mass cytometry.

Aim 2: To determine the efficacy of the first-generation P2X7R antagonist, PKT100 to reduce inflammation in PBMCs of patients with MI and CAD.

Chapter 4

Hypothesis: PKT100 can effectively ameliorate atherosclerotic plaque instability through selective P2X7R antagonism.

Aim 1: To determine the efficacy of PKT100 in stabilising atherosclerotic plaque in a murine model of plaque destabilisation.

Chapter 5

Hypothesis: Immunomodulation through PKT100 may confer substantial benefit through P2X7R antagonism in a murine model of obesity-driven cardiometabolic syndrome.

Aim 1: To evaluate whether PKT100 can mitigate diastolic dysfunction and myocardial alterations driven by obesity independent of changes in body weight and metabolic parameters in an experimental murine model.

Chapter 6

Hypothesis: The development of a novel series of P2X7R antagonists may elicit more potent immunomodulatory activity than first-generation P2X7R antagonists.

Aim 1: To determine the in vitro potency of a novel series of cyanoguanidine-adamantane hybrids against IL-1 β and IL-18 secretion

Aim 2: To demonstrate the effectiveness of the series in reducing IL-1 β secretion from participants with unstable and stable CAD, in combination with benchmarking compounds.

Aim 3: To determine the in vitro potency of novel fluorinated adamantane-amide P2X7R antagonists against IL-1 β and IL-18 secretion.

Aim 4: To demonstrate the effectiveness of the series in reducing IL-1 β , IL-18, and IL-6 secretion from participants with unstable and stable CAD.

Chapter 2

General Methods

Method overview

This chapter offers a broad overview of the general methods applicable to this thesis. Detailed procedures are outlined in the subsequent manuscript-style chapters. This chapter intends to provide context for the experimental strategies utilised, briefly discussing the inherent advantages and limitations of the applied methods. Notably, the evaluation of the first-generation P2X7R antagonists in vivo prompts critical enquiry into the applicability of CVD models that aim to bridge the gap in translation between preclinical models and human clinical trials. Moreover, a thorough discussion is presented in this chapter on the critical questions addressed through the utilisation of the animal models in the research.

In vitro experiments

The THP-1 cell line is a spontaneously immortalised human monocytic cell line derived from the peripheral blood of a young patient with acute monocytic leukemia. (1) The cell line is significant in P2X7R drug discovery due to its endogenous expression of the P2X7R. (2) The THP-1 line is commercially available and provides a background for consistent and uniform evaluation of pharmacological agents. Under non-stimulated conditions, the cell line is non-adherent and grown in suspension in RPMI-1640 (ATCC modified medium, supplemented with low sodium bicarbonate, L-glutamine, sodium pyruvate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, phenol red, and high glucose) (Gibco, USA). The medium was supplemented with 10% fetal bovine serum (FBS) (AU origin, Gibco) in a T175 cell culture flask (Corning, USA). When stimulated with interferon-gamma (IFN- γ) and lipopolysaccharide (LPS), THP-1 cells differentiate into activated M1-like macrophages. (3) Treatment with IFN- γ increases adhesion molecules ICAM-1 and vascular cell adhesion protein-1, which aids macrophage adhesion to polyethylene plates. (3) LPS, a constituent of the outer membrane of gram-negative bacteria, also elicits transient adhesion of THP-1-activated macrophages through tyrosine phosphatase activation of β 2-integrin. (4) Activating integrins enhances adhesion between cells and extracellular matrix proteins. (4) LPS also stimulates the toll-like receptor-4 complex, where its activation is associated with NF- κ B translocation and production of pro-inflammatory cytokines and differentiation of monocytes into M1-like macrophages. (5) Thus, stimulation with IFN- γ and LPS is required for cellular diversification, cellular adhesion, and pro-inflammatory cytokine expression.

Under these treatment conditions, compound evaluation is relatively robust and can be achieved in a concentration-dependent manner. For this reason, THP-1 cells have been widely used in immunology, and their capacity to secrete cytokines has made them foundational to the evaluation of compounds with immunomodulatory benefits. From the perspective of CVD, macrophages have a distinct role in atherosclerosis development and progression, where macrophages are associated with vascular adhesion and transmigration

into the subendothelial space. (6–9) The THP-1 cell line, stored in liquid nitrogen, was used to maintain consistency throughout experiments. Cells were thawed at passage 23 and were used until passage 48, when their proliferative capabilities began to decline. Cells were thawed in a water bath at 37°C until only a thin layer of ice remained. The cells were then aspirated into a T175 culture flask with 20 mL of supplemented RPMI-1640 and 10% FBS. Cells were maintained in a 37°C incubator enriched with 5% CO₂ and passaged every two days. On experimental days, at least 3 x 10⁶ cells were returned to a new flask to continue cellular propagation.

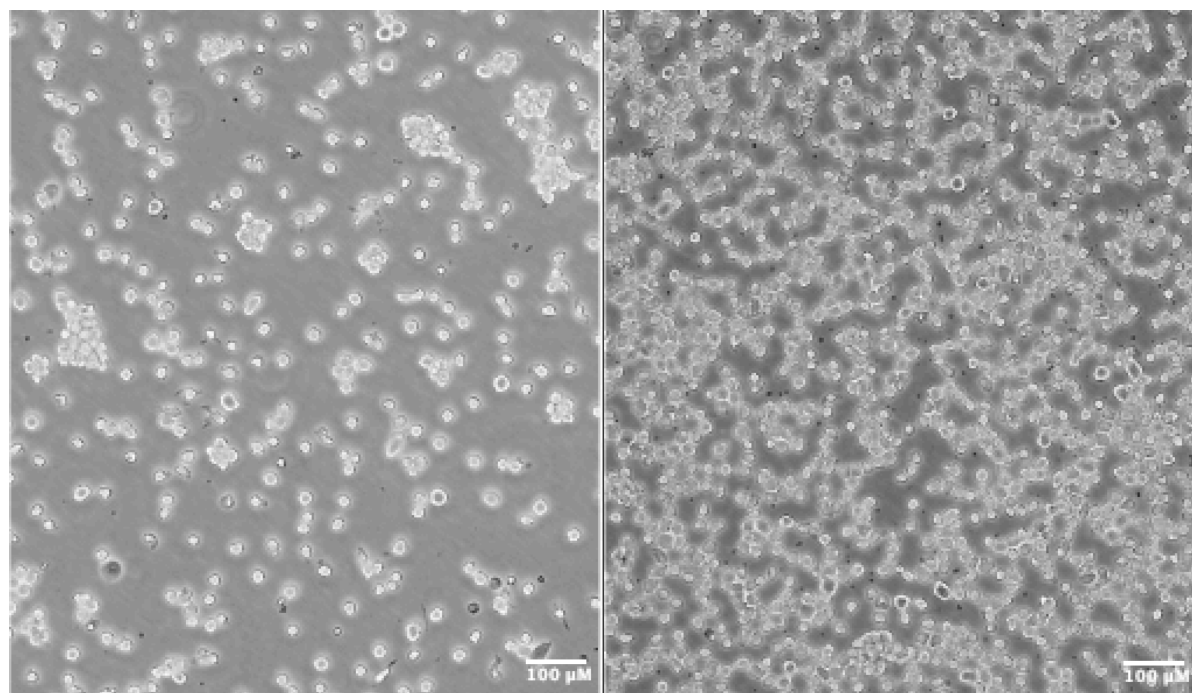


Figure 1. THP-1 cells cultured for in vitro high-throughput screening of P2X7R antagonists against pore-formation and cytokine release. Cells are demonstrated to form aggregates in their healthy state and separate into single cell forms when stressed. **Images adapted from ATCC.**

Compound reconstitution for in vitro assessment.

The design and synthesis of the first-generation P2X7R antagonist, PKT100, was achieved by Dr Shane Michael Wilkinson and Professor Michael Kassiou from the School of Chemistry University of Sydney. The compound was previously selected from a panel of 70 adamantane benzamide analogues due to its potency in pore-formation assays and improved metabolic stability compared to the initial adamantane amide lead compound. (10) The details of its design and its assessment in vitro are published elsewhere. (11) The second-generation P2X7R antagonists reported in this thesis were synthesised by Dr James O'Brien-Brown and Mr Jayson Gilchrist under the supervision of Professor Michael Kassiou. This series of compounds incorporated the cyanoguanidine motif previously reported by Abbott Laboratories and the adamantane-based moiety, which was reported to increase metabolic stability, as seen for PKT100. An initial panel of adamantly-cyanoguanidine hybrids was reported and influenced the further development of adamantanyl-cyanoguanidine analogues reported in this thesis. (12)

Mr Jayson Gilchrist and Professor Michael Kassiou developed an additional fourth-generation P2X7R antagonist. The moiety for these compounds was based on the scaffold reported by AZ. (13) This series intended to increase the metabolic stability of compounds by modifying the adamantanyl amide linker. This strategy was previously explored for the adamantanyl benzamide series that led to the successful development of PKT100. The initial potency evaluation of the adamantanyl-cyanoguanidine and -amide scaffolds using Yo-Pro-1 dye uptake assay was conducted by Mr Andre McKenzie under the supervision of Dr Eryn Werry and Professor Michael Kassiou at the School of Science University of Sydney. The details of these experiments are addressed in Chapter 6. Compounds with a half-maximal inhibitory concentration (IC_{50}) of < 80 nM were selected for further in vitro and ex vivo pharmacological characterisation. Compounds were evaluated against commercially available P2X7R antagonists and selected based on their established potency in vitro. (13–16) These compounds were previously explored in clinical trials and served as a reference in the ex vivo assay. All compounds selected were determined to have an ideal pharmacokinetic and safety profile based on first-in-man clinical studies. Benchmarking compounds AZD9056, GSK-1482160, and JNJ-5175446 were purchased from MedChemExpress (USA).

The selective P2X7R agonist BzATP was selected to activate the P2X7R. It is described as a synthetic ATP analogue with increased specificity and potency at the P2X7R. (17) The stock of BzATP from MedChemExpress and diluted it at a concentration of 5 mM in UltraPure™ DNase/RNase-Free distilled water (Sigma-Aldrich, USA) on arrival. 90 μ L aliquots of BzATP were stored at -80°C for the study and thawed 10 minutes before use. For in vitro and ex vivo experiments, all compounds were reconstituted at 5 mM in pure dimethylsulfoxide (DMSO, Sigma-Aldrich) and stored at -80°C for the duration of the experiments. Compounds were thawed 30 minutes before experiments, and any remainder of the aliquot was discarded after the experiment.

Concentration-response experiments.

Various assays have been employed in P2X7R drug development to characterise the pharmacological properties of novel compounds. Assays that measure pore formation with Yo-Pro-1 dye (Thermo Fisher Scientific, USA) uptake are extensively used due to their reliability and simplicity. This assay harnesses the molecular capabilities of the P2X7R to form macropores upon activation. (17) The binding of ATP induces Yo-Pro-1 dye permeation through the membrane, where it irreversibly binds with nucleic acids to increase fluorescent intensity. Compounds are assessed on their potency to diminish pore formation in a concentration-dependent manner. However, pore formation mechanisms may not consistently align with IL-1 β release. Thus, a pipeline to characterise the novel series of P2X7R antagonists in vitro, enabling the selection of lead candidates.

Immune cells play an intricate role in disease intensification by releasing cytokines that bind to extracellular cytokine receptors to modulate cross-cellular communication. (18) The small regulatory cytokines, IL-1 β and IL-18, are accentuated as notable contributors in atherosclerosis and plaque rupture. (19–21) Given the crucial role of the P2X7R in the maturation and release of cytokines, an IL-1 β release immunoassay was used to determine compound efficacy in a concentration-dependent manner. However, several limitations exist in this experiment,

where adequate and uniform macrophage adhesion is essential. A 96-well polyethylene plate coated with CellBIND (Corning) patented formula was used to enhance macrophage adhesion and control for this limitation. The increased hydrophilic surface achieves better cell adherence with non-adherent cell lines in serum-free conditions.

Once cells were estimated to have reached a rate of exponential replication, cells were aspirated into a 50 mL centrifuge tube (Corning) and centrifuged at 300 $\times g$ for 5 minutes at room temperature with the break on. The supernatant containing waste and cellular debris was removed, and the pellet was resuspended in 5 mL of RPMI-1640 without FBS. Ten microlitres of the resuspended pellet was removed and diluted 1:1 with 0.4% trypan blue solution (Gibco) to be counted. A valid count was achieved by averaging the number of cells counted with a hemocytometer and the number of cells counted automatically with the Countess 3 automated cell counter (Thermo Fisher Scientific). The remainder of the cells were held in an upright position at 37°C until the cell count was complete. Cells were then reconstituted at a density of 2×10^5 cells per 100 μL and treated with 10 ng/mL recombinant human IFN- γ (carrier-free, R&D Systems, USA) and 25 ng/mL LPS from E.coli strain (0111:B4, Sigma-Aldrich). From this step, it is crucial that cells are not incubated in medium containing FBS, as the presence of FBS substantially influences BzATP-dependent P2X7R activation. (22) Treated THP-1 cells were then seeded into a 96-well, Corning CellBIND flat bottom transparent microplate (Corning) at a density of 2×10^5 cells per well at a volume of 100 μL and incubated for 3 hours at 37°C in CO₂ enriched incubator.

After incubation, the supernatant was removed from the wells and cells were pre-treated with either (i) 0.001% DMSO if cells were to remain untreated or (ii) P2X7R antagonists resuspended in RPMI-1640 at a concentration between 0.001 - 1 μM for 30 minutes at 37°C. Pre-treatments were removed, and cells were gently washed with 150 μL of phosphate-buffered saline (PBS) without magnesium and calcium (pH 7.4, osmolarity 270-300 mOsm/kg, Gibco). Under experimental conditions, PBS used for washes and incubation should be free of magnesium and calcium as these ions are known to allosterically modulate the potency of BzATP at the receptor. (22) PBS was then gently aspirated, and cells were treated with either (i) 0.001% DMSO in unstimulated basal wells, (ii) 1 mM BzATP diluted in 0.001% DMSO to stimulate cytokine release through the P2X7R, or (iii) 0.001 – 1 μM P2X7R antagonists in combination with 1 mM BzATP. The plate was incubated for 1 hour at 37°C. After treatment, the supernatant was collected and transferred to a and centrifuged for 5 minutes at 125 g at room temperature. The supernatant was then collected into a new microcentrifuge tube (Eppendorf, Germany) to eliminate potential contamination from cellular debris. Samples were stored at -80°C until subsequent downstream analysis.

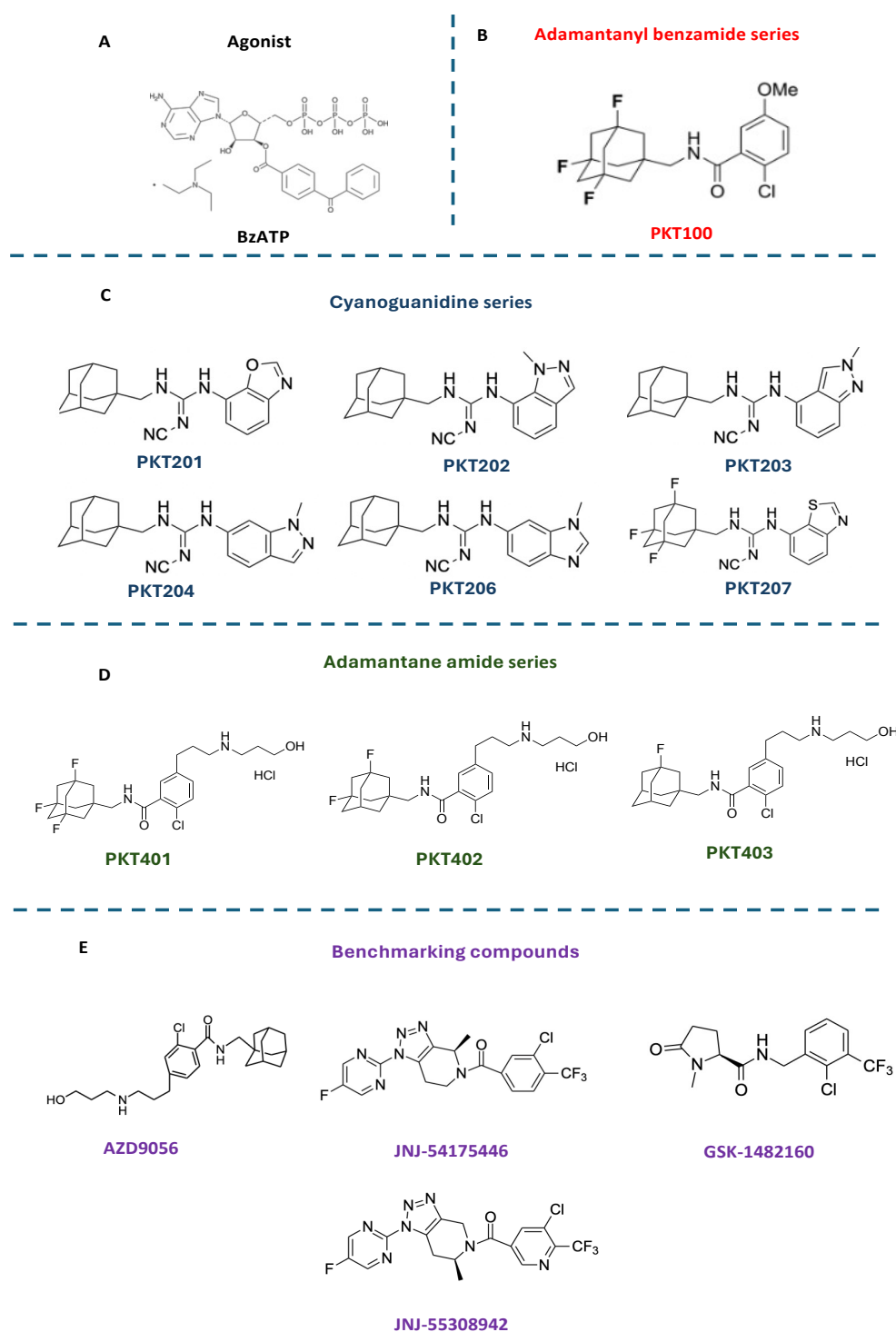


Figure 2. Structures of newly developed compounds aimed to target the P2X7R and ATP analogue BzATP. A) Depicted in black is the agonist structure, BzATP. **B)** Depicted in red is the first-generation lead adamantanyl benzamide compound, PKT100. **C)** Depicted in blue are the six analogues of the lead cyanoguanidine series compounds. **D)** Depicted in green are the three analogues of the lead adamantane amide series compounds. **E)** Depicted in purple are the four benchmarking compounds evaluated alongside the novel series.

In vitro potency assessment

To evaluate cytokine secretion from PBMCs, enzyme-linked immunosorbent assays (ELISA) was used to quantify protein concentrations. Human IL-1 beta/IL-1F2 and total IL-18 monoclonal capture antibodies were coated onto high-bind 96-well clear, flat-bottom polystyrene microplates (R&D Systems) overnight at room temperature. Plates not used immediately the next day were stored at 4°C. Before sample preparation, plates were washed with 0.05% Tween 20 in PBS, pH 7.2-7.4 diluted in Milli-Q water three times, with the solution removed between each wash. To prevent the non-specific binding of protein to the microplate, wells were then blocked with 0.1% BSA in PBS (R&D Systems) prepared in PBS (referred from here on as 'blocking buffer') for 2 hours at room temperature. Samples from in vitro experiments are thawed at room temperature and then placed on ice before preparation to avoid protein degradation. Samples were reconstituted 1:2 to detect IL-1 β and 1:1 to capture IL-18 in the prepared blocking buffer and incubated for 2 hours at room temperature. After these samples were aspirated, wells were washed with 0.05% Tween 20 in PBS, as detailed above. A second antibody aimed to detect the attached protein, commonly called the 'detection' antibody (R&D Systems), was then incubated in the wells for 2 hours at room temperature. After this time, the additional detection antibody was washed away as detailed above, and a 1:40 streptavidin (R&D Systems) conjugated with horseradish-peroxidase enzyme solution diluted in blocking buffer was included in each well for 20 minutes at room temperature. To remove unbound streptavidin (R&D Systems), the plate was again washed as detailed above, and a 1:1 solution of H₂O₂ and tetramethylbenzidine (R&D Systems) was added to the well at room temperature for 20 minutes. This solution interacts with bound horseradish-peroxidase enzyme to induce a colourimetric conversion on a spectrum from clear to dark blue. The colour change depends on the amount of IL-1 β , or IL-18 detected in the well. The reaction is stopped with 2N H₂SO₄, and the amount of specific protein in the sample was detected on a microplate reader (Varioskan LUX, Thermo Fisher Scientific) by measuring the amount of light absorbed by the reagent at a 450 nm wavelength with a 540 nm wavelength correction.

In vitro potency analysis

The analysis for this component of experiments was completed in GraphPad Prism software 10.0 (GraphPad Software Inc, USA). For pre-processing normalisation, raw absorbance values were exported into CSV format. Compensation for well-to-well variations was achieved by the following equation: 450 nm value – and 540 nm value. Variation in these measures was corrected by averaging biological replicates to correct for pipetting errors. The background signal from each well was corrected by the following equation: standard or unknown wells – blank well. Normalised data was then imported into GraphPad Prism, where the absorbance values for the standard equated with the corresponding predetermined concentration in an XY table. Unknown values were copied below for interpolation. To achieve a normal distribution, values were log-transformed (base 10) and then interpolated as a nonlinear regression, where the bottom was constrained to 0. For prospective analysis, values were untransformed with the equation $X=10^X$. At this stage, normalised and interpolated concentrations were exported back to Excel to calculate concentration-dependent reductions in IL-1 β , and IL-18 release. The interpolated concentrations were processed to reflect the concentrations reached by adjusting for

the dilution factor of the sample in the well. To derive the percentage reduction in IL-1 β or IL-18, the basal concentration was removed from all other concentrations and the following equation was applied:

$$\frac{(\text{normalised BzATP concentration} - \text{normalised sample concentration})}{\text{normalised BzATP concentration}}$$

These derived values were then subjected to statistical evaluation in GraphPad Prism software, where the percentage reduction was plotted compared to the concentration-dependent logarithmic concentration. A nonlinear regression analysis was performed with a four-parameter variable slope to obtain IC₅₀.

PBMC Isolation

Peripheral blood mononuclear cells (PBMCs) are critical components of the immune system, playing an important roles in both innate and adaptative immune responses against invading pathogens. (23) Moreover, the adaptation of immune populations to respond to persistent inflammation, driven by cytokines and antigens in patients with ASCVD, may hold important insights for the detection and treatment of CAD. (24,25) Current diagnostic strategies for advanced subclinical atherosclerosis rely on stratification for CT coronary angiography (CTCA) based on established risk factors. Unlike in cancer diagnosis, where tissue biopsy enhances diagnostic and therapeutic precision, in atherosclerosis, biopsy of plaque is rare, and the cellular contribution to the disease remains poorly understood. (26) Circulating PBMCs offer a potential advantage over traditional biopsies, as they are easily accessible from the blood, where they interact with stimuli and ligands that activate them, together with atherosclerotic lesions.

The development of high-dimensional single-cell methods has revolutionised the comprehension of these populations in atherosclerosis and highlighting the link between heightened inflammation with the progression of ASCVD. (27) Fernandez et al., 2020, published the most comprehensive atlas on immune cells using paired plaque and blood samples from patients with ASCVD. (28) In a more recent study, the same team utilised Cytometry by Time-of-Flight (CyTOF) and RNA-seq to identify an inflammatory transcriptional signature from PBMCs. (27) This approach resulted in the identification of a list of small molecules predicted to attenuate inflammation through these pathways. (27) Saracatinib was selected as a lead candidate and was shown to exert anti-atherosclerotic effects in a murine model of atherosclerosis. (27) From a drug discovery perspective, PBMCs from patients with ASCVD provide a physiologically relevant model to assess compound efficacy. Moreover, their use in this setting provides a novel approach to exploring patient-specific differences, such as compound effectiveness in the presence of genetic variations.

To evaluate PBMCs from the selected population, the Ficoll-gradient method was employed to separate PBMCs from other blood cells. Whole blood was collected by venous cannulation into vacuette lithium heparin tubes (Greiner Vacuette, Austria). Whole blood was diluted 1:1 with Hank's Balanced Salt Solution (HBSS) with calcium chloride and magnesium chloride (Thermo Fisher Scientific) and gently inverted three times. Diluted whole blood was layered on top of 13 mL of Ficoll-paque medium (density 1.077 g/mL, Cytiva, USA) in a 50 mL centrifuge tube and centrifuged at 800 $\times g$ for 15 minutes at room temperature with the break-off. As per Figure 3, samples

removed from the centrifuge were separated based on density, with the PBMCs forming the interphase between the plasma and the Ficoll-mixed granulocytic region, effectively removing polynucleated immune populations such as neutrophils and erythrocytes. PBMCs were collected into a 15 mL from the interphase region into centrifuge tubes and centrifuged at 800 $\times g$ for 10 minutes at room temperature with the break on low, minimising erythrocyte contamination. After carefully removal of the supernatant, PBMCs were resuspended by gently swiping the side of the tube and resuspending in 10 mL of HBSS with calcium and magnesium. The sample was inverted gently three times, and ten microlitres of resuspended PBMCs were removed and diluted 1:1 with 0.4% trypan blue for counting. As mentioned above, for in vitro experiments, a valid cell count was determined by averaging the total cells counted manually using a haemocytometer and the total cells counted using the Countess 3 automated cell counter (Thermo Fisher Scientific). The centrifugation process was completed a second time at 800 $\times g$ for 10 minutes at room temperature with the break on low. The wash buffer was removed, and PBMCs were resuspended at 10×10^6 cells/mL in RPMI-1640 with 2 mM L-glutamine but without heat-inactivated bovine serum (HIBS, Thermo Fisher Scientific) for downstream stimulation experiments. Serum-free conditions were employed to enhance monocyte adhesion. A proportion of all PBMCs underwent cryopreservation for CyTOF experiments. PBMCs were resuspended in freezing medium containing 90% HIBS and 10% DMSO and slowly brought to -80°C in a cryo-freezer (Thermo Fisher Scientific) until transferred to liquid nitrogen.

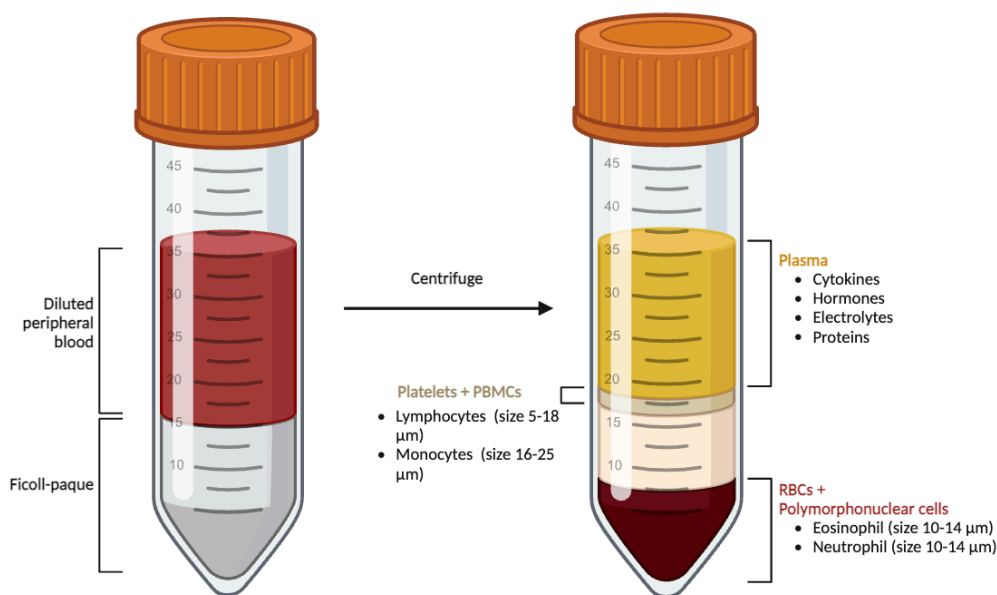


Figure 3. Depiction of PBMC isolation method. Diluted blood is layered on Ficoll-paque and centrifuged to separate blood based on density. The PBMC layer is located between the plasma layer and the polymorphonuclear cells. Created with Biorender

Participant population

Participants were recruited to the BioHEART trial approved by the Northern Sydney Local Health District Human Research Ethics Committee (HREC/17/HAWKE/343). BioHEART was established to facilitate the discovery of

markers and mechanisms of subclinical atherosclerosis. (29) Participants undergoing CTCA were recruited. A thorough history was gathered from each participant. A trained physician performed a CACS assessment, which is used in this thesis to stratify patients. CTCA were analysed according to the standard anatomical arterial regions defined by the 17-segment model outlined by the Society of Cardiology Cardiovascular Computed Tomography. (30) A secondary assessment of CT images was completed at the School of Health Sciences University of Sydney, led by Professor Stuart Grieve. Further analysis reconfirmed that CACS was calculated using the Agatston method and was scored based on the degree of stenosis and composition of the plaque. (29). Moreover, Gensini scores were quantified for each segment, recording the degree of stenosis. (26) The CACS percentile, which aims to stratify patients based on their 10-year CVD event risk, was determined by incorporating age and sex into the calculation. Additionally, participants were included who underwent percutaneous coronary intervention for STEMI. Demographic information was limited to information gathered upon admission through the emergency department at Royal North Shore Hospital. Detailed descriptions of both studies have been published elsewhere. (29)

Ex vivo patient-derived monocyte assay

PBMCs derived from patients with active disease states have proven indispensable for therapeutic and biomarker discovery. The field of biomarker discovery has undergone rapid transformation, whereby the manipulation of PBMC activation has enabled better discrimination of disease severity. (31) Under stimulated conditions, PBMCs from patients with ASCVD treated with atherosclerotic plasma demonstrate heightened secretion of pro-inflammatory cytokines IL-1 β and IL-6. (27) LPS-stimulated PBMCs from patients with acute coronary syndrome have also showed exacerbated inflammatory signatures in those without standard modifiable risk factors. (30) Under these circumstances, existing methods were adapted to measure pro-inflammatory cytokine release related to P2X7R activation. Further adaptation was made to evaluate the efficacy of the panel of P2X7R antagonists in influencing cytokine secretion in a disease-relevant model. Compared to previous methods, enriched monocytes were not treated with IFN- γ and LPS as previously described methods. Instead, physiological concentrations of cytokine release from monocytes were assessed.

After resuspension at 10×10^6 cells/mL in RPMI-1640, PBMCs were plated in duplicate in a 96-well flat bottom CellBIND (Corning) microplate at a density of 1.6×10^5 cells/well at a volume of 100 μ L. Monocyte enrichment was achieved through a brief 3-hour incubation at 37°C, where monocytes readily adhere to the treated polystyrene microplate. After this, the medium was removed from the wells and replaced with compounds reconstituted in RPMI-1640 for 30 minutes at 37°C. A single test concentration of 200 nM of each P2X7R antagonist was chosen based on the median reported IC₅₀ from initial *in vitro* compound characterisation experiments. It was hypothesised that several of the P2X7R antagonists would exhibit maximal inhibition at this concentration while less potent compounds would elicit weaker responses in inhibiting cytokine release. The diversity of their inhibition would help to identify P2X7R antagonists with improved potency. Pre-treatments were then aspirated, and cells were washed gently with 150 μ L PBS without calcium and magnesium to remove

any potential interference from RPMI-1640 or non-adherent populations. Cells were then treated with the compound in combination with BzATP for 30 minutes at 37°C as indicated in the respective chapters (referred to in detail in Chapter 3 and 6 methods). The supernatant was collected into a clean microcentrifuge tube (Eppendorf) and centrifuged at room temperature at 800 $\times g$ for 5 minutes. Supernatants were then transferred to a clean microcentrifuge tube (Eppendorf) to eliminate any potential contamination from cells carried over, and samples were stored at -80°C until required for downstream analysis.

ProQuantum assessment of cytokines from monocytes

For decades, the quantification of cytokines from supernatants has relied on antibody-based ELISA, which is generally costly and lacks specificity. Despite some newer immunosorbent kits that greatly reduce the time to detection, traditional ELISA require a considerable volume to achieve sufficient detection. To overcome these limitations, ProQuantum high-sensitivity immunoassays were utilised for the detection of cytokines in a high-throughput manner. Compared to traditional systems, ProQuantum immunoassays (Thermo Fisher Scientific) enable the detection of proteins with greater sensitivity and lower sample consumption in a 384-well format. Detection is facilitated through proximity ligation technology, combining antigen-antibody interaction for protein detection with quantitative PCR (qPCR) to amplify the signal. (33) Target-specific antibodies that contain two different epitopes for the same protein are conjugated with 3' and 5' prime oligos. (33) The adhesion of the epitopes to the analyte brings the 3' and 5' prime regions in close proximity, where the oligos form a bridge through incubation with a DNA ligase. (33) Heat inactivation produces single-strand DNA of approximately 100 base pairs proportional to the initial protein-bound concentration, serving as the template for amplification.

Supernatant samples from PBMC were thawed at room temperature and immediately placed on ice after thawing. To achieve concentrations within the standard curve, samples were diluted 1:1 in assay dilution buffer prepared from the ProQuantum kit (Thermo Fisher Scientific). The protein standard (Thermo Fisher Scientific) was similarly diluted in dilution buffer to the recommended volume and left to stand at room temperature for 15 minutes. A cocktail that contained antibody conjugates A and B, diluted in the required conjugate dilution buffer (Thermo Fisher Scientific), was used to facilitate the binding of the analytes from the supernatant. Samples were prepared in a 1:1 ratio with analyte binding solution (Thermo Fisher Scientific) in duplicates, sealed, vortexed briefly, and centrifuged at 3000 $\times g$ for 1 minute at room temperature. To achieve antibody-protein adhesion, the plate was incubated at room temperature for 1 hour. After this time, a master mix containing the DNA ligase and thermostable DNA polymerase (Thermo Fisher Scientific) was prepared according to the kit instructions. The master mix was dispensed in multichannel format into each well, after which the plate was sealed with an opti-seal for PCR, vortexed briefly and centrifuged at 3000 $\times g$ for 1 minute at room temperature. The plate was read in a 384-well format on a 12K QuantStudio Flex Real-Time PCR System (Thermo Fisher Scientific). Experiment parameters were configured for standard curve or quantitation using TaqMan reagents. The reporter dye was set to FAM, with the quencher reported as NFQ-MGB and the passive reference as ROX.

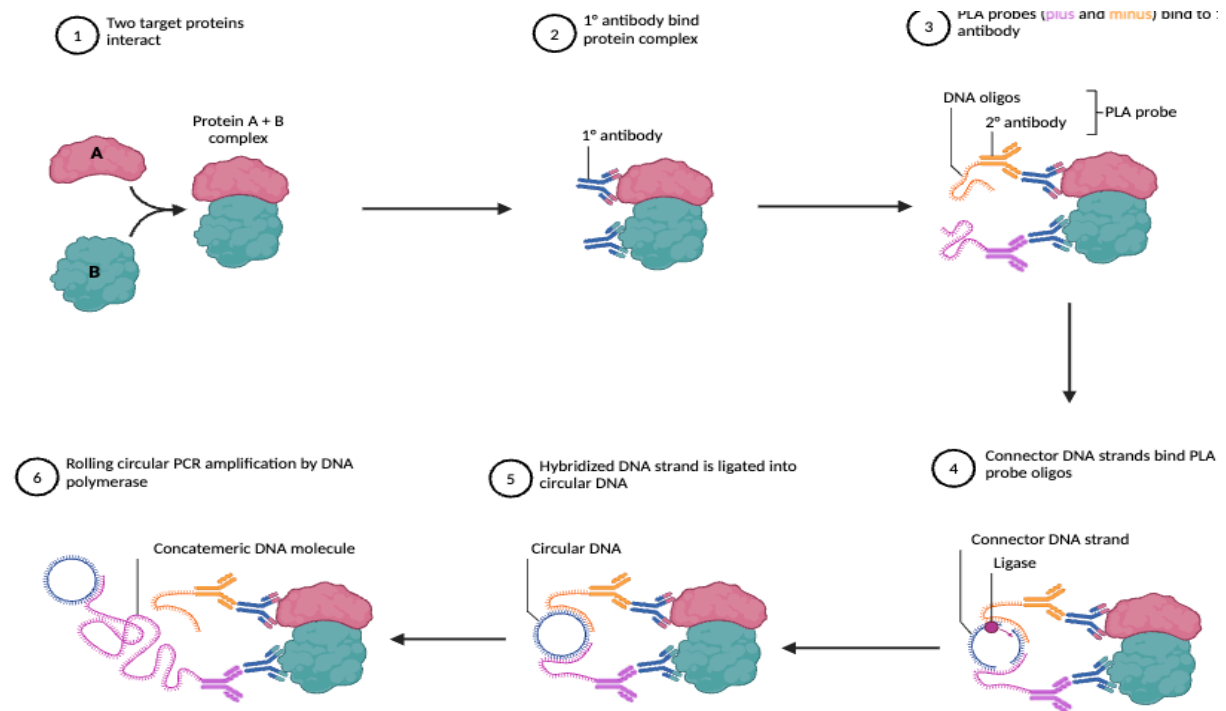


Figure 4. Visual representation of the molecular mechanisms underlying proximity ligation assay. This technique uses two independent antibodies conjugated with DNA oligos that bind to different epitopes on the same protein. Upon adhesion, the close proximity of the DNA oligo in the presence of a ligase results in the formation of circular DNA. The connector serves as the product template for the TaqMan polymerase. The detection of the specified sequence results in new strands of DNA. A fluorescent signal is released each time the dye is separated from the quencher. This indicates that the polymerase has cleaved the TaqMan probe. **Created with Biorender.**

ProQuantum Analysis

Data were exported as a CSV file. Duplicates from the run were averaged. Any CT values further than 1 CT away from each other were assessed for the true CT score based on other replicates. For interpolating unknown values in a standard curve, values were copied into Prism 10.0 for further analysis. Values were log-transformed (base 10) to achieve normal distribution. A variable slope with four parameters was established to interpolate the log-transformed variables into the standard curve. No adjustments were made to the bottom and top values. Concentrations in picograms per mL were achieved by reversing the transformation of these values. Percentage reduction in IL-1 β , IL-18, and IL-6 release was derived using the formula reported above in the section on 'In vitro potency assessment'.

CyTOF

The characterisation of immune populations into distinct subtypes depends on the availability of advanced detection tools capable of comprehensive analysis on a single-cell level. CyTOF integrates the principles of flow cytometry and mass spectrometry to facilitate the detection and quantification of immune populations. (26) Antibodies labelled with rare earth metal isotopes are incubated with PBMCs, where the mass of heavy metals is computationally coded for a particular marker. (34) This method allows for a deeper understanding of immune

populations in ASCVD. The research presented in this thesis was conducted in collaboration with Dr Helen McGuire from the School of Medical Sciences University of Sydney. For human samples detailed in Chapter 3, PBMCs were stored in a freezing medium containing 90% HBS and 10% DMSO. After thawing, cells were washed with RPMI-1640 and counted with a hemocytometer. PBMCs were stained with 1.25 μ M cisplatin (Sigma-Aldrich) in PBS for 3 min at room temperature to identify dead cells. PBMCs were then resuspended in FACS buffer (Biolegend, USA) supplemented with 5% FBS. PBMCs were then incubated for 30 minutes at 4°C with a cocktail of metal-conjugated antibodies targeting cell surface antigens, with specific antibody details provided in the methods section in Chapter three. Cells were then fixed and permeabilised with FoxP3 buffer kit (eBiosciences, USA) at 4°C for 45 minutes and stained with intracellular antibodies for 30 minutes on ice, with antibody details also provided in Chapter 3. PBMCs were stained with a DNA intercalator (0.125 μ M Iridium-191/193; Fluidigm, USA) to discriminate live single-nucleated populations. After several wash steps, cells were filtered through 35 μ M nylon mesh (Thermo Fisher Scientific) and diluted with equilibrium beads. The equilibrium beads were used to normalise the signal intensity using CyTOF Software v7.1 for Helios. FlowJo X 10.0.7r2 software (FlowJo, USA) was used to gate populations of interest, export median signal intensity for markers across populations, and study participants for samples with a minimum of 1×10^4 live cells to analyse.

CyTOF Analysis

After normalising the median signal intensity for each immune population, the sample identification number was used to integrate the P2X7R expression intensity across classical monocytes, non-classical monocytes, B cells, CD1c⁺ mDCs, CD141⁺ mDCs, plasmacytoid dendritic cells (pDCs), and natural killer (NK) cells. An initial analysis of variance (ANOVA) was conducted to assess the percentage of cells expressing P2X7R expression, with comparisons made between binary variables no CAD vs. CAD, and no CAD vs STEMI. The same analysis was also conducted for median intensity of P2X7R expression. For multiregression analysis, clinical parameters such as CACS, SPS and Gensini, which can take infinite values and exhibit zero inflation due to no CAD controls reported as zero, were adjusted using a log (base 10) + 1 transformation to achieve a normal distribution for analysis. A Pearson correlation test was performed to assess the relationship between each variable and immune populations, specifically examining whether P2X7R intensity was associated with certain risk factors. Additionally, it was important to delineate potential differences in demographics and risk factors between the no CAD vs. CAD, and no CAD vs STEMI groups, in order to identify covariates for adjustment in multivariate regression analysis. Multivariable regression models were then constructed to assess the relationship of P2X7R intensity across immune populations and clinical parameters, with clinical outcomes as the dependent variable. For visualisation, the results were presented as partial regression plots, and R² values and p-values from the regression analysis were reported. Statistical significance was defined as a p-value of less than 0.05.

Preclinical studies

Animals

All experiments with small animals were conducted according to the Australia's National Health and Medical Research Council (NHMRC) guidelines and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The Animal Ethics Committee of the University of Sydney, Australia and the Alfred Research Alliance, Australia, approved all animal experiments. 6–9-week-old male ApoE^{-/-} mice were acquired from the Australian Resources Centre (Australia). Male C67BL/6J wild-type mice reported in Chapter 4 were obtained from the Animal BioResources (Australia). Male C67BL/6J wild-type mice reported in Chapter 5 were acquired from the Kearns Facility at the Kolling Institute of Medical Research (Australia). For experiments, all mice were acclimatised for at least one week in the holding room before the commencement of the protocol. Mice were housed in groups of at least two for the duration of the experiments. The facility was maintained at an optimal temperature of 22°C, with a 12-hour light/dark cycle in line with typical mouse nocturnal activity. Mice were provided with water and food ad libitum unless specified in specific chapters.

Preclinical models of CVD

Genetic manipulation of cholesterol pathways to evaluate atherosclerotic plaque development in small animals.

It was traditionally hypothesised that atherosclerosis results from dysregulated lipid transport, leading to the retention of lipids in intimal arterial walls. (35) Particularly, epidemiological studies identified early on that hypercholesterolemia or excessive serum cholesterol was associated with the development of CAD. (36) This association was further strengthened by identifying an autosomal dominant variant of hypercholesterolemia resulting from defects in the gene that encodes for the LDL-R. (37) While advances in genetic testing now recognise more than 900 mutations in this LDL-R gene, the most common attributable characteristic associated with genetic defects in LDL-R is heightened LDL cholesterol in serum. (38) Indeed, these blood results manifest from the inability of LDL to be removed from the circulation. The clearance of LDL is dependent on the internalisation of particles and the degradation in lysosomes. (39) This process is mediated by the LDL-R, which binds LDL to promote lysosome degradation. Independent of other risk factors, those with familial 32wsahypercholesterolemia exhibit a heightened risk for premature CAD and is a significant predictor for MI. (40) Comparatively, mice tend to be relatively resistant to the development of atherosclerosis due to substantial differences in lipid metabolism compared to humans. (41) Of particular importance, mice are determined to exhibit lower concentrations of LDL compared with high-density lipoprotein (HDL). This phenomenon could be explained by the non-detectable cholesteryl ester transfer protein activity levels. (42) Cholesteryl ester transfer protein promotes the transfer of HDL 'good' cholesterol into ApoB-containing LDL and is determined to be a crucial contributor to atherogenesis. (42)

ApoE was recognised as a crucial regulator of lipoprotein clearance, particularly emphasised for its atheroprotective function. (43) ApoE is further distinguished into either ApoE2, -3, or -4 isoforms, attributed to

amino acid modifications in the N-terminal domain. (44) These isoforms, characterised by their intrinsic affinity for the LDL-R, function to various extents to remove LDL from the circulation. (45) For instance, the prevalence of ApoE2 is renowned for its role in atherosclerosis, facilitated by higher unbound plasma ApoE, equipped to bind free lipids. (46) Indeed, the importance of this isoform is evident from its close genetic association with lipoprotein(a). (47) Lipoprotein(a) concentrations, which are largely genetically determined, denote susceptibility to CAD, independent of other risk factors. In contrast, the expression of the ApoE4 allele has long been known for its association with Alzheimer's disease and atherosclerosis. (48) The stronger affinity of ApoE4 for LDL-R compared with ApoE2 isoforms diminishes its capabilities to remove LDL due to heightened premature occupancy of the receptor. (45)

Genetic exploitation of these pathways in animal models has been fundamental in atherosclerosis research. LDL-R^{-/-} mice (B6.129S7-Ldlr^{tm1Her}/J(002207)), similar to human mutations, demonstrate dysregulated lipid transport, accumulating LDL in circulating plasma. Their use in atherosclerosis research is extensive, although the mice are limited in generating atherosclerotic plaques without implementing a high-cholesterol, atherogenic diet. (49) Comparatively, ApoE^{-/-} (B6.129P2-Apoetm1Unc/J(002052)) mice exhibit extensive and rapid atherosclerotic plaque lesions with heightened LDL concentration. (50) The rapid development of atherosclerotic plaques with unstable plaque features has popularised the strain in atherosclerosis research, although plaque development is suggested to be rather heterogeneous.

Moreover, atherosclerotic plaque progression can be accelerated by treatment with atherogenic diets or diabetes induction with streptozotocin. By comparison, 'humanised' and 'Western' diets high in saturated fats are shown to substantially elevate plasma LDL concentrations in LDL-R^{-/-} mice with only a modest effect on atherosclerotic lesion development. (41) Either way, both mutations modify lipid transport for the reasons described above. However, even under genetic manipulation, LDL-R^{-/-} and ApoE^{-/-} mice fail to develop atherosclerotic plaques in the coronary tree, with lesions more likely to form in the proximal aorta and brachiocephalic trunk. (51) Comparatively, humans more frequently develop lesions in the coronary arteries, carotids, and iliac vessels. (41) Considering these details, the ApoE^{-/-} was the selected variant due to the substantial development of atherosclerotic lesions with unstable, measurable features.

Towards a more translational preclinical model of atherosclerotic plaque instability

The development of murine models that mimic human atherosclerosis is crucial for assessing novel pharmacotherapeutics. While current models, such as those in ApoE^{-/-} mice, have helped assess features of stable atherosclerotic plaque, they fail to resemble advanced atherosclerotic lesions. Thus, the development of animal models that more closely mimic human disease is widely sought after in atherosclerosis and drug discovery research. As explained above, common atherosclerosis research models depend on the genetic deletion of lipid transport genes. (51) Even in ApoE^{-/-}, rapidly developing atherosclerotic lesions, complex and advanced lesion features such as intraplaque haemorrhage and thrombosis are rare (52). There is a disconnect

between humans and other mammals, where plaque destabilisation is the most significant cause of ischemic vascular events that lead to mortality. To address this unmet need in preclinical research, a murine model, termed 'tandem stenosis', aimed to mimic the features of plaque instability in humans was developed. Like in human atherosclerosis, the induction of two ligations on the right carotid artery (RCA) reduces shear stress and increases tensile stress, resulting in features of plaque destabilisation. (52) Particularly in segment 1, which is affected by the turbulence of blood flow, atherosclerotic plaque in ApoE^{-/-} mice is characterised by intraplaque haemorrhage, neovascularisation, and luminal thrombi. (52) Moreover, the plaque in this region is largely acellular, indicating the presence of large lipid-rich necrotic cores, aside from the abundance of inflammatory infiltrates. (52)

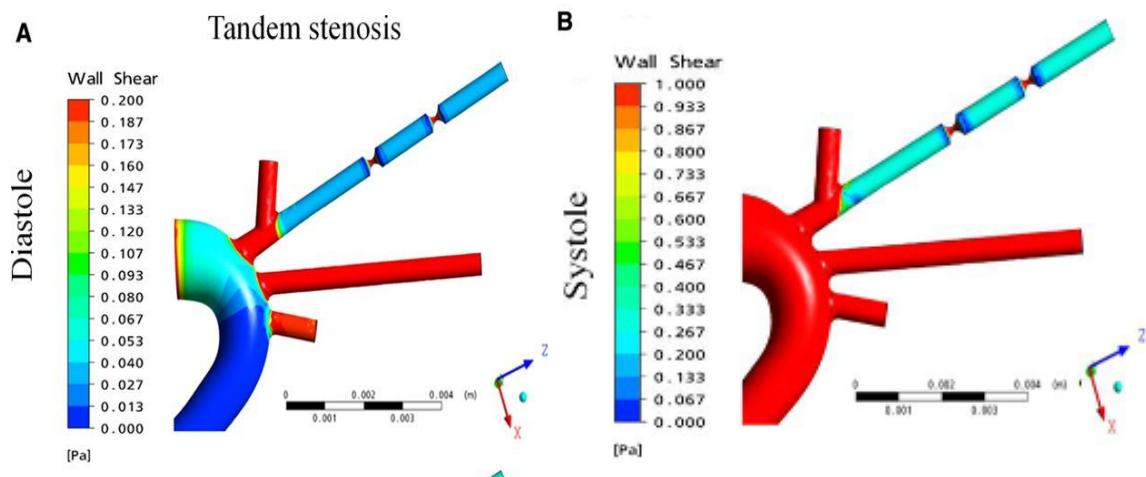


Figure 4. Depiction of the computational fluid dynamics in the tandem stenosis murine model. A) Wall stress is indicated to be significantly reduced in diastole. **B)** Wall stress is indicated to be significantly reduced in systole. (52)

In the protocol, mice were acquired at 6-9 weeks of age and receive an HFD starting from 7 – 10 weeks. After this 7-week timeframe, mice are anesthetised with a combination of ketamine 100 mg and xylazine 10 mg. The neck is opened, and the submandibular glands are gently separated to access the muscularised region. The RCA artery is identified and isolated from surrounding connective tissue. In careful succession, surgical ligation of the right carotid artery 1 mm below the bifurcation and 4 mm proximal to this ligation is induced with 8-0 silk. The region was sutured back together, and the mice were revived with atropine after the procedure. Under isoflurane, a lateral cut was made in the interscapular region and undermined to create a subcutaneous pocket for micro-osmotic minipump (Alzet model 1004 0.16 μ L per hour for 28 days, Durect Corporation USA) inserted to deliver 0.2 mg/kg/day of PKT100, or vehicle for the remaining 6 weeks before euthanising. A detailed description of the preparation and use of this passive device is provided in this thesis under 'micro-osmotic minipump preparation'.

At the time of tissue collection, the arterial system is divided up into five segments for consecutive analysis. Segment I is referred to as the unstable segment. It is characterised by intraplaque haemorrhage, luminal thrombosis, acellular necrotic core, lipid accumulation and a disrupted fibrous cap. (52) Comparatively, segment II, just distal to this region, has an intact fibrous cap with high cellularity. Segment III is an intact, stable plaque segment with a highly fibrous cap. Segment IV is the left carotid artery and is characterised as plaque-free. Segment V comprises the aortic arch and contains stable atherosclerotic plaques.

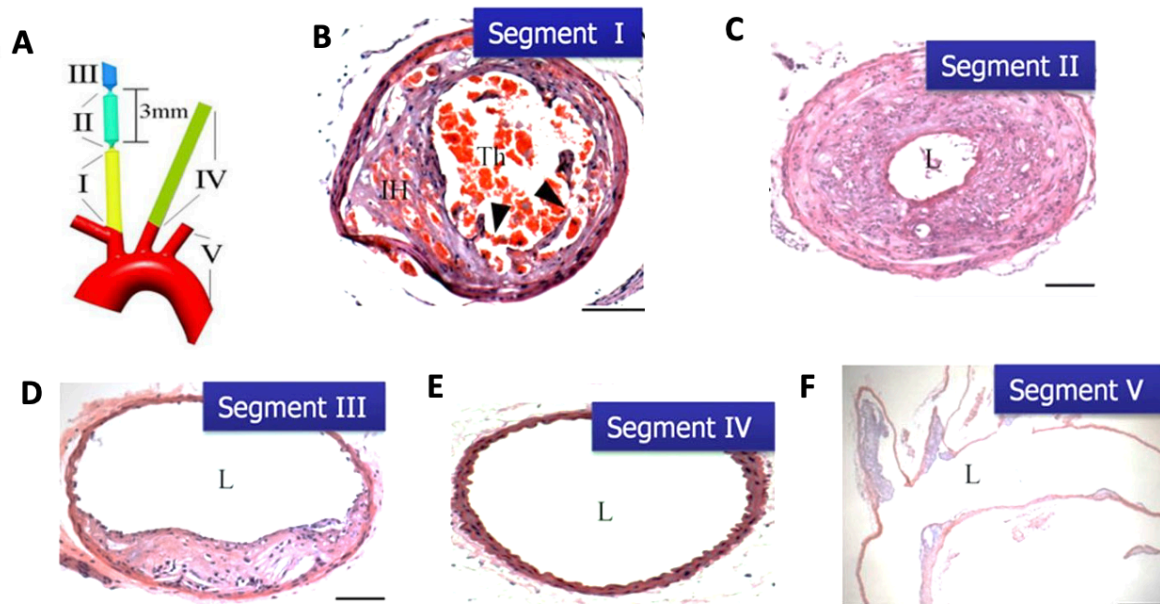


Figure 5. Representation of vessel segmentation and characterisation in the tandem stenosis model. A) Depicts the vessel segmentation in the intact vessel with segment I closer to the bifurcation of the innominate artery and segment III most distal. **B-F)** Characterisation of the intravascular plaque phenotype associated with each segment. (52)

Assessment of atherosclerotic plaque destabilisation

Post-mortem studies after acute MI events have played an essential role in the characterisation of unstable plaque phenotype. Despite this, post-mortem lesions indicate plaque morphology following an event, where the characteristics of unstable phenotypes are altered following plaque rupture and thrombosis. (53) Advancements in imaging methods, such as optical coherence tomography, have enabled finer resolution in the characterisation of plaque morphology before an event. (54) Radiographic assessment of atherosclerotic lesions with optical coherence tomography or non-invasive intravascular ultrasound has shown that lipid-rich necrotic core size correlates with potential future CVD events. (55,56) Treatment with high-intensity statins was shown to decrease intraplaque lipid burden. (55) The analysis evaluated lipid-rich intraplaque content in Oil red O (ORO), a dye that stains neutral triglycerides and lipids in tissues. However, the stain has limitations as it is most effective in fresh preserved samples, given that alcohol solvents remove the majority of lipids. (57) For this reason, all experiments completed for this model used unfixed, flash-frozen samples.

Intraplaque haemorrhage have been detected in rupture-prone atherosclerotic plaque, where intraplaque haemorrhage is also associated with increased neovascularisation (**Figure 6**). (58,59) The hypoxic conditions induce immature neo-vessel formation, where erythrocytes leak into the plaque and promote inflammation. (59,60) Intraplaque haemorrhage expression was measured through TER119, a surface marker for erythrocytes. TER119 expression was measured based on the percentage of pixels positive within a plaque area. To detect neovascularisation, CD31, a surface marker was used to distinguish endothelial cells. Regions positive for CD31 were quantified through total pixels positive for CD31 stain within a plaque region. Diversification of VSMCs is an extensive topic of its own. VSMCs are suggested to differentiate into macrophage-like cells in plaque to elicit atheroprotective and inflammatory events. (61,62) Indeed, their expression in stable atherosclerotic plaques is associated with the deposition of collagen that strengthens the extracellular matrix. At the same time, their later transmigration to the fibrous cap is linked with plaque destabilisation and rupture. Moreover, VSMCs are suggested to drive the development of microcalcifications, known as spotty calcification, that elicit weaknesses in lesions. (63,64) As described above, plaque VSMC expression was measured with the intracellular marker α -smooth muscle actin (α -SMA). A polarised lens was used to capture collagen deposition and fibrosis cap thickness with a picrosirius red (PSR) stain.

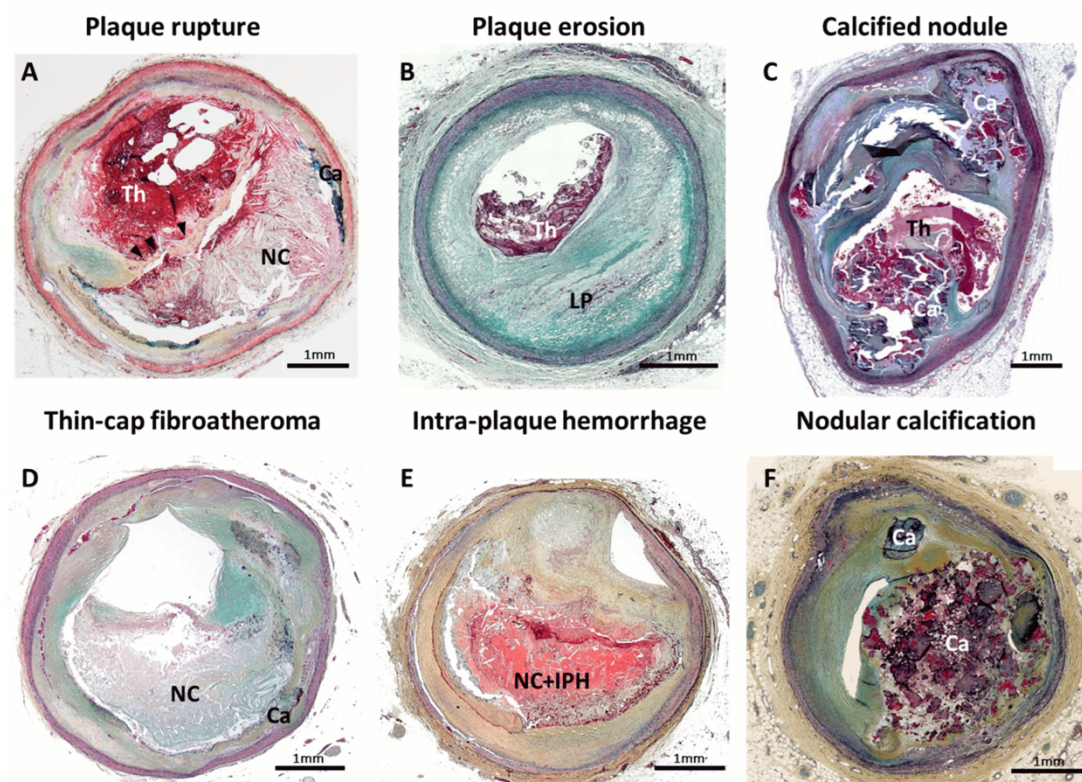


Figure 6. **A)** Histological representation of a plaque rupture with characteristic thrombus formation and a large necrotic core. **B)** A plaque with characteristics of erosion indicated by a large lipid-depleted necrotic core. **C)** A ruptured plaque with calcification. **D)** An atherosclerotic plaque with a thin fibrous cap covering a large necrotic core. **E)** A plaque with a large region of intraplaque haemorrhage. **F)** Ruptured plaque with large regions of nodular calcification. (65)

Plasma lipids and inflammatory markers

Previous human trials have shown that the anti-inflammatory benefits of Canakinumab in patients with a residual risk could be achieved independently of a modification in plasma lipids. (20,66) Despite this, reducing lipids with statins has also demonstrated a potent anti-inflammatory benefit in human trials. (67,68) Thus, in mice studies in this thesis, plasma cholesterol, LDL, HDL, and triglycerides were evaluated to understand whether PKT100 may elicit a therapeutic benefit on inflammation independent of plasma lipid reduction. Plasma lipids were measured on a Cobas Integra 400 plus autoanalyzer (Roche Diagnostics, Germany) following the standard commercial enzymatic assay kits. Colorimetric changes were measured by 512 nm wavelength for total cholesterol and triglycerides and 583 nm for HDL and LDL. These measures were completed simultaneously.

The efficacy of PKT100 in reducing systemic inflammation was evaluated using multiplex Luminex technology (Thermo Fisher Scientific). The ProcartaPlex immunoassay utilises capture antibodies conjugated to magnetic beads corresponding to a distinct spectral code. After incubation with plasma diluted 1:1 with universal dilution buffer, a detection antibody aimed at the analyte creates a sandwich-like structure akin to a standard ELISA. The magnetic beads are washed between incubations by attaching a magnetic plate washer to ensure magnetic beads adhere to the bottom of the wells. Wells were washed by quick inversions, where 150 μ L of wash buffer was added to each well for 30 seconds before the plate was quickly inverted and blotted on an absorbent towel. Beads are then incubated with streptavidin-R-phycoerythrin (Thermo Fisher Scientific), which carries a fluorescent wavelength and uses biotin binding principles to differentiate between several targets simultaneously. Following a final wash step, the beads are resuspended in a universal resuspension buffer for analysis. The sample is aspirated using flow cytometry and immobilised beads with a magnetic force. The bead colour and fluorescence intensity indicate the analyte that is excited by light-emitting diodes captured on a charge-couple device camera which is an essential component of the MAGPIX analyser (Diasorin, USA). The raw values exported from this experiment are interpolated in a standard curve with predefined concentrations to derive the plasma concentrations of inflammatory markers.

Assessment of the inflammatory contribution to obesity-driven HFpEF

Clinical manifestations of HFpEF have been difficult to recapitulate in small animals that are relatively protected against diastolic dysfunction. (69) This is reflected in the scarcity of effective pharmacotherapeutics for patients with HFpEF. (70) Additionally, the multitude of different phenotypes of HFpEF attributed to aetiology has resulted in the expansion of animal models which aim to emulate distinct pathogenesis. (71) Current models are characterised as (i) single factorial, (ii) two-, or (iii) three-hit, where the contribution of individual risk factors is tested alone and in combination with other risk factors. (71,72) Notably, for the evaluation of pressure-overload hypertrophy in HF, TAC that introduces a ligation between the innominate and left carotid artery is preferred as a consistent and efficient model of HF within 4-weeks. (73) Conventional HFD models aimed to imitate the pathophysiological changes associated with obesity and insulin resistance are relatively straightforward approaches to induce HFpEF related to cardiometabolic disease. (74) A significant limitation of this model is the

(75) Age is a considerable contributor to HFpEF, where age-related alterations in MI are also linked to a higher prevalence of extra-cardiac impairments. (71) This includes substantial kidney, skeletal muscle, and lung impairments due to systemic age-related changes. (71) However, aged murine models also come at the disadvantage of time and cost, particularly if purchased externally. Amongst these models, female mice exhibit a milder phenotype compared to age-matched male mice (76) despite the HFpEF being more common in older human females. (77) While the reasons for these sex differences are poorly understood, some studies propose that oestrogen may confer benefits in younger female mice (78), although this remains contentious. For this reason, male mice were exclusively utilised in this study due to significant logistic issues associated with sourcing aged female mice.

While the pathogenesis of HFpEF is linked with chronic and gradual disease progression, animal models with multifactorial drivers, known as two- or three-hit models, aim to accelerate the clinical presentation of the syndrome. (71) The short timeframe required to observe symptoms of HFpEF in a two-hit HFD and L-NAME model has gained prominence in the literature. (79) Within these studies, male mice demonstrate adverse changes in diastolic and myocardial deformation as early as five weeks after initiating the experiment. (79) Given the immediate severity and multifactorial contribution in the two-hit model, an obesity model was selected to evaluate the efficacy of PKT100 to protect against adverse LV remodelling, independent of other confounding variables. 12-week-old C57BL/6J male mice initiated an HFD composed of 45% digestible energy from lipids for 24 weeks. Mice were provided with food and water ad libitum unless otherwise specified. Metabolic parameters were assessed through weekly non-fasted glucose measurements obtained via tail-prick in mice. Heart function was observed with echocardiography at baseline and 4, 8, 12, 16, 20, and 24 weeks after commencement. As described above, mice received continuous delivery of 0.2 mg/kg/day of PKT100, or vehicle via micro-osmotic minipump from week 16 until the completion of the study. The heart was separated into segments based on anatomical distinct regions. The LV was characterised as the region of interest and was carefully dissected from the RV and atria. The left ventricle was then dissected transversely at the level of the papillary muscles, where the inferior portion was frozen cut side down in Optimal Cutting Temperature (Sakura Finetek, USA) for histology. The superior portion was cut into smaller portions and frozen for molecular assessment. Measures of cardiomyocyte hypertrophy were reported based on fluorescent wheat-germ agglutinin (WGA) stain. PSR was used to delineate measures of interstitial fibrosis.

Micro-osmotic minipump preparation

For all in vivo models reported in this thesis, PKT100 was diffused through manual osmotic displacement, which is driven by differences in the pressure between the minipump compartment and the tissue where it is implanted. (80) This delivery method is beneficial for extended treatment protocols as the drug concentration is maintained at a pre-determined rate, negating the requirement for repeated oral gavage or intraperitoneal injections. Particularly for compounds with poor pharmacokinetics, delivery through osmotic minipump infusion allows a mechanism to be established without initial complex chemistry synthesis. Given that the pump is

implanted subcutaneously, this allows for direct systemic delivery and minimises potential adverse events that could result through daily oral or intraperitoneal interference.

Briefly, micro-osmotic minipumps were filled as described in accordance with the manufacturer's instructions. Based on previous pharmacokinetic studies reported in WO2020037350A1 that described the synthesis and use of PKT100, a concentration of 0.2 mg/kg/day was established for in vivo models that utilised micro-osmotic minipump delivery. (81) Due to its initial development as a blood brain barrier permeating compound, PKT100 was determined to be lipophilic and demonstrate poor solubility in hydrophilic solutions such as saline. For this reason, to achieve adequate solubility PKT100 was reconstituted in a 50% DMSO, 35% saline, and 15% ethanol solution. Micro-osmotic minipumps were inserted into mice with a total volume of 100 μ L to be delivered over 28 days at 3.84 μ L per day. Mice treated with vehicle received a minipump that contained the same vehicle composition without the drug. While the total volume of DMSO is not ideal for delivery in mice due to the potential toxicity associated with its infusion, delivery through micro-osmotic minipumps limits the volume of DMSO to 1.92 μ L per day. Additionally, to avoid any issues, the day prior to implantation, micro-osmotic minipumps were filled with their respective treatment and primed overnight in sterile 0.9% saline at 37°C. Any osmotic minipumps that floated in saline were refilled to remove air bubbles to ensure adequate drug perfusion. For protocols that require treatment beyond 4 weeks, minipumps were replaced to ensure continuous delivery of PKT100.

Echocardiography

Small animal echocardiography is a gold-standard approach for consistent and non-invasive heart function measurements. Although several challenges persist in image acquisition, given the small size of mice hearts coupled with high heart rates, the development of high-frequency instruments with improved high frame rates has significantly enhanced echocardiography in small animals. A crucial aspect often overlooked is the importance of appropriate anesthesia to capture physiological measures. Unlike in humans, mice are restrained during echocardiography, which, without anesthesia, is stressful and leads to variations in diastolic measurements and image acquisition. (82) For this reason, for the studies, which utilised echocardiography (FUJIFILM VisualSonics, Canada) isoflurane was delivered at 1 to 1.25% at a rate of 1 litre/minute. The physiological parameters such as heart rate, body temperature, and respiration rate were monitored throughout the procedures to confirm image acquisition was performed within physiological ranges. Body temperature was measured using the VisualSonics rectal probe, where temperatures between 36 to 37.5°C were maintained with exogenous heat lamps (Morganville Scientific, USA). To prevent mice from overheating, heat lamps were placed to warm the air in the room instead of directly on the rodent. The VisualSonics heat pad that the mouse was stationed on was also set at 37°C to help with body temperature maintenance. Parameters of body temperature are important determinants of heart function. (82)

Heart rate was monitored by the VisualSonics electrocardiogram, with murine paws positioned on the animal platform equipped with electrodes. Under isoflurane, heart rate was maintained between 450-550 beats per minute (BPM) to acquire systolic measures. Recent literature has aimed to standardise echocardiography in rodents and provide a detailed guideline to achieve reproducible measurements of cardiac function. (82) As part of this guideline, Zacchigna and the ESC Working Group on Myocardial Infarction propose that diastolic parameters should also be captured at heart rates above 450 BPM. (82) While there is a general agreement with the literature, for assessment of diastolic dysfunction, particularly measured as the E/A ratio, heart rate should be reduced to visualise the separation of the E and A peak. For non-invasive assessment of LV filling pressure measured by E/e', heart rate can remain above 450 BPM, although the peaks captured are a fusion of the E and A waves. This limitation has been addressed in the diastolic parameters by ensuring that heart rate was consistent between experimental groups. The electrocardiogram-derived respiration was evaluated, and the respiration rate was maintained between 150 – 190 during image acquisition.

Image Acquisition

Systolic function

Systolic function and LV size were captured in B- and M-mode in the parasternal long (PSLAX) and short axis (PSAX). Visualisation of the apex, together with the aortic root, to ensure consistency in heart function in the PSLAX (**Figure 7A**). These regions were aligned in the PSLAX, where the apex was juxtaposed to the aortic root. The anterior papillary is present in this orientation, providing a landmark for M-mode acquisition. In the PSAX, the heart was positioned so that the anterior papillary muscles were prominent and provided an anatomical landmark for image acquisition (**Figure 7B**). The transducer was shifted 90°, where the LV took on a rounded shape from the appearance of the interventricular septum. Image acquisition was achieved with the Vevo3100 imaging system (VisualSonics Inc, Canada) equipped with a 40-MHz probe (MS400). The cursor was positioned after the papillary muscles for M-mode acquisition for consistency. Physiological measures of ejection fraction, fractional shortening, cardiac output, and stroke volume were reported. Anatomical measures such as LV mass, diameter in systole and diastole, and the left anterior wall thickness were obtained.

Diastolic function

Diastolic measures were acquired in doppler modes in the four-chamber view. (82) The four-chamber plane constitutes the RV and LV separated by the septum on top of the right and left atrium (**Figure 8A**). The platform was inclined 50° backwards and slightly to the right to obtain the four-chamber view, positioning the mouse head down towards the bench surface. The transducer was angled to the right, similar to the position of the probe in the PSAX and aligned with the tip of the apex through the subcostal space. (83) Image acquisition in power doppler mode facilitated by colour doppler assessment of flow velocity. Blood flow towards the transducer, indicated by a positive doppler shift, was marked in red, while movement away from the source, indicating a negative doppler shift, was marked in blue. (82) The probe was aligned with the flow direction from the atrium to the LV, capturing changes in filling velocity as a key determinant in HFpEF. The direction of the

sample volume, which is pointed in the direction of the flow, is significant for these measures and facilitates image acquisition with high velocity. **Figure 8B** shows sample volume acquisition at these regions of high velocity, which depicts the relationship between LV filling and the cardiac cycle.

To assess measures of diastolic function, flow velocity was measured as indicated by previous studies. (82) Under baseline conditions, the E wave exhibits the highest velocity, representing passive blood flow from the left atrium into the LV. (82) The E wave is particularly influenced by conditions that affect myocardial relaxation and, thus, increase ventricular pressure, impairing the passive flow of blood from the left atrium in diastole. (84,85) The left atrial pressure is increased to maintain LV preload and to counteract the increased pressure in the LV in diastole. (86) The A wave represents the active flow of blood from the atrium into the LV during the contraction phase. (85) Under normal physiological conditions, the velocity of the A wave is lower, accounting for the 15-20% of blood moved into the LV after atrial contraction. (85) Disparities in A wave velocity are governed by changes in compliance and contractility of the left atrium. (82) The isovolumetric contraction time denotes the contraction of the ventricle, where the pressure is relieved through the no-flow phase. A slight increase in pressure is detected in the isovolumetric relaxation phase. Patients with HFpEF can exhibit a prolonged isovolumetric relaxation phase, indicating an inability of myocardium relaxation. (87)

To evaluate diastolic function, mitral valve motion was measured using tissue doppler mode. The ratio between E/e' is used as a tool for the assessment of diastolic function and is obtained from the E wave acquired through power doppler analysis and the E wave from the tissue doppler mode (**Figure 8C**). The E/e' is proposed to be a critical measure for the clinical diagnosis of HFpEF and represents LV filling pressures. (82) Moreover, isovolumetric contraction and isovolumetric relaxation were measured in tissue doppler mode. Measures of mitral valve motion were acquired in the tissue doppler mode, where the sample volume collected was positioned on the anterior mitral annulus with the pointer directed towards the apex. The heart rate again decreased below 450 BPM to consistently obtain the E' and A' peaks. The disadvantages of this have already been noted above. Future HFpEF studies completed should capture all physiological measures at a heart rate above 450 BPM. Under these circumstances, changes in diastolic parameters will be reported from the E/e' , together with other measures.

Speckle-tracking echocardiography (strain)

The degree of myocardial deformation is measured by tracking acoustic markers generated by the myocardium in the systolic and diastolic phases. (88) Comparatively to conventional echocardiography, subtle abnormalities can be detected by strain, particularly in the setting of cardiomyopathies. (89) In the PSLAX view, the endo- and epicardium are traced, and the total deformation is measured as the length change between the relaxation and contraction phases. (90) This is referred to as the global longitudinal strain (GLS), which has demonstrated predictive value for mortality in patients with EF > 35%. (90) The value of GLS in this important cohort was determined to be independent and cumulative with EF. (90) In conjunction with diastolic parameters, the GLS

has been applied in preclinical studies due to its ability to detect variations in myocardial deformation before the reduction in systolic parameters. (79,91) Mice typically exhibit a GLS of approximately -20% under normal physiological conditions, a measurement comparable to humans. (91–93)

In humans, strain is acquired by averaging each myocardial segment recorded from the longitudinal, circumferential, and radial views. (94) Comparatively, mice studies that report strain primarily report GLS in a single plane. This is due to the poorer resolution associated with mouse echocardiography, which is further limited by the size of the heart. Image quality is an important consideration when tracing the endo- and epicardium. For models of MI in mice, this makes strain analysis particularly useless, given that a substantial part of the endocardium is lost after necrosis. Any assessed measures may not accurately reflect myocardial displacement from this single-view plane. strain measures also benefit from determining myocardial deformation in the circumferential and radial view. In Chapter 5, global circumferential strain was used to evaluate deformation from the PSAX and provide confirmation for the GLS values. This was conducted by tracing both the endo- and epicardium at the level of the papillary muscles. The papillary muscles were not included as a feature in the analysis to ensure that deformation was not attributed to the papillary muscles. For future studies, measures of strain will follow human guidelines closer to derive better predictions of myocardial function.

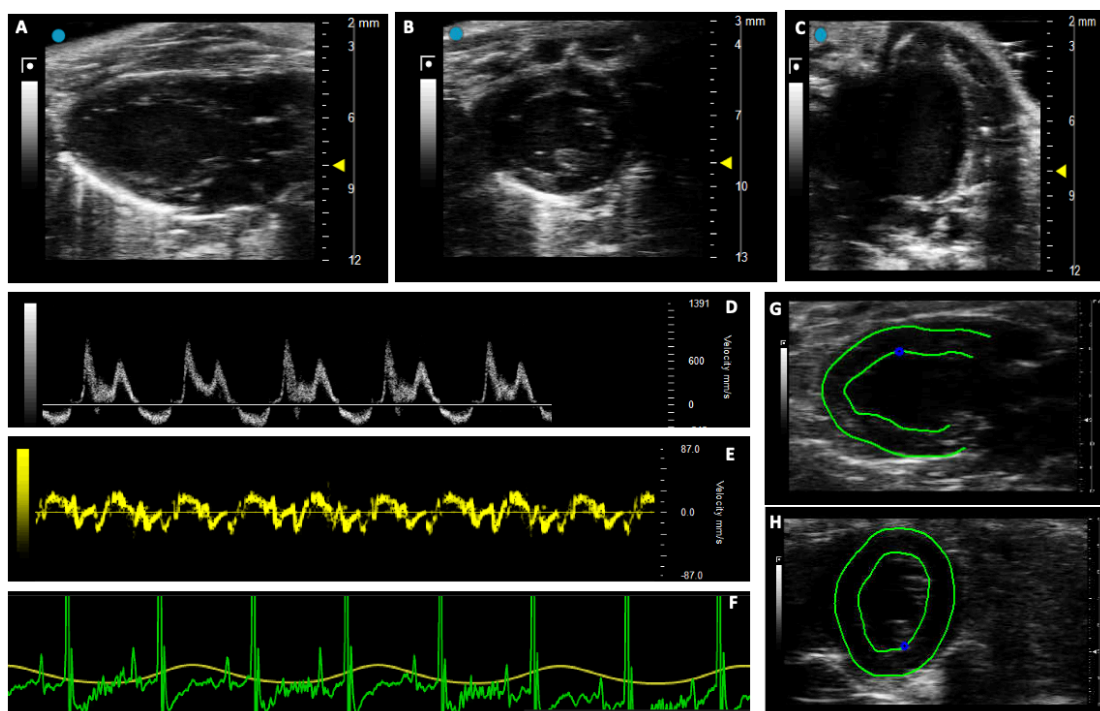


Figure 7. A representation of the mouse heart is depicted with 2D ultrasound on echocardiography. A) The heart is represented in the PSLAX position with the characteristic aortic root juxtaposed to the apex. **B)** The heart is represented in the PSAX position with the characteristic anterior papillary muscles and rounded shape. **C)** The heart is represented in the four-chamber view with the characteristic mitral valve and intraventricular septum. **D)** Representation of the pulse-wave Doppler recording with the peak flow velocities indicated by the E and A peaks. **E)** Representation of the mitral tissue Doppler recordings with the peak flow velocities indicated by the

E' and A' peaks. **F)** Representation of the ECG collected through echocardiography with overlapping breath waves. **G)** Representation of the global longitudinal strain analysis. **H)** Representation of the global circumferential strain analysis.

Histology

Several histological techniques were used throughout the results chapters to evaluate features associated with the disease. Each chapter extensively covers the details of the tissue collection and preparation methods. Histology was generally performed on fresh frozen tissues embedded in OCT medium on an NX70 cryostat (Thermo Fisher Scientific). Samples were cut between 7 μ m allowed to air-dry on slides for at least 30 minutes at room temperature to achieve adequate adhesion. Samples were stored unfixed at the -80°C until staining took place.

Hematoxylin and Eosin

Samples were removed from the -80°C and brought to room temperature for one hour on the bench. Prior to staining for hematoxylin and eosin, 7 μ m sections were fixed in ice-cold acetone (Sigma-Aldrich) for 10 minutes. After this time, the acetone was left to evaporated from the slides for 30 minutes at room temperature. Slides were incubated in hematoxylin (Sigma-Aldrich) at room temperature for 5 minutes and then washed under running water for 5 minutes. Slides were incubated in Scott's solution (Sigma-Aldrich) for 1 minute at room temperature to differentiate the nuclei. The slides were rinsed quickly in running water and dabbed carefully to remove excess water. Slides were then transferred to eosin (Sigma-Aldrich) for 15 seconds to prevent dye over-saturation. Eosin stains the extracellular matrix and should show a medium pink colour contrasting with the blue/purple nuclei. Slides were washed a final time in running water and then underwent a quick dehydration procedure in four changes of histology-grade 100% ethanol and followed by three changes in xylene (Point of Care Diagnostics, Australia). Slides were mounted with a histology-grade Eukitt resin (Sigma-Aldrich).

PSR

The extracellular matrix is a complex, interconnected network of proteins primarily composed of collagen, elastin, proteoglycans, and glycoproteins. (95) Atherosclerotic lesions with an unstable phenotype are characterised by the degradation of extracellular matrix proteins such as collagen. (53) Secondary preventatives such as statins restore collagen composition and stabilise plaque to the detriment of plaque volume. (96,97) However, this is a primarily attributed feature to calcified plaques compared with noncalcified soft plaques. (97) Comparatively, the increased deposition of interstitial fibrosis in the myocardium is associated with adverse structural and functional alterations in the heart. For the detection of collagen, PSR is used to enhance the natural birefringence of collagen fibres. (98) The anionic structure of PSR binds to cationic collagen I and III bundles and is detected with either light microscopy or under polarised light. (98) While light microscopy is the most common method for fibrosis visualisation, polarised light improves collagen detection due to its representation on a dark background.

Collagen is commonly detected in tissue fixed in formaldehyde and paraffin-embedded, increasing protein cross-linking. For the studies in this thesis, unfixed frozen mouse tissue was used since the exact replicates could be used for immunofluorescent staining and RNA extraction from off-cuts. Samples were removed from the freezer and allowed to come to room temperature for 1 hour. Fixation was achieved depending on the imaging required for analysis. In the case of the heart sections, acetone (Sigma-Aldrich) fixation was sufficient to achieve a clear differentiation between other components of the extracellular matrix and collagen. For the plaque segments, formaldehyde fixation was used as samples were evaluated under a polarised lens. Detection of collagen fibres was enhanced with 4% formaldehyde fixation (Sigma-Aldrich) under polarised light. Slides were allowed to dry for 20 minutes at room temperature before being submerged in preprepared Bouin's solution (Sigma-Aldrich) for 30 minutes. After this time, slides were moved directly into the PSR solution made from 0.5% w/v fast red 80 dye (Sigma-Aldrich) and reconstituted in 1.3% saturated aqueous picric acid (Sigma-Aldrich) for 30 minutes at room temperature. Samples were differentiated by running the slides under distilled water and dabbing on a paper towel to remove water. Slides were dehydrated in four changes of ethanol, followed by four changes of xylene (Point of Care Diagnostics), before being mounted with a histology-grade eukitt resin (Sigma-Aldrich).

Immunohistochemistry

Using immunohistochemical procedures for tissue provides an advantage for the long-term storage of stained samples. Compared to immunofluorescence, where samples lose their intensity over time, 3,3'-diaminobenzidine-detected samples are stable at room temperature indefinitely. Like the above protocols, samples were thawed and brought to room temperature for at least 1 hour. Samples were fixed in ice-cold acetone and subsequently blocked with a blocking buffer containing 1% BSA in PBS for 30 minutes at room temperature. Primary antibodies were reconstituted in the same blocking buffer and incubated on samples for one hour at room temperature to achieve protein detection. The antibodies used were anti-CD31 (clone MEC13.3; 1:100 dilution; R&D Systems; anti-SMA (clone 1A4; 1:100 dilution, Sigma-Aldrich); rat anti-mouse CD68 (MCA1957GA; 1:100); and TER119 (13-5921-81; 1:400, eBioscience). Detection was achieved using the Vectastain ABC (Thermo Fisher Scientific) kit and 3,3'-diaminobenzidine (Thermo Fisher Scientific) substrate. The specificity of rat antibodies was achieved with rat IgG2B control to validate the stain. Other isotype control antibodies were used to validate each immunostaining according to the primary antibody. All images were captured on an Olympus BX41 (20x objective) using an Olympus DP22 camera.

Immunofluorescence

Compared to standard immunohistochemical approaches, immunofluorescence microscopy is more sensitive and specific, enhancing the detection capabilities. Moreover, the simultaneous detection of several proteins within the same tissue section is made possible by using multiple fluorophores emitted at a distinct wavelength and conjugated to specific antibodies. Beyond the visualisation of protein patterns, fluorescent microscopy provides insights into the localisation and interaction of proteins within their spatial context. Slides were brought to room temperature for 1 hour minimum after removed from the -80°C. Slides were fixed at room temperature

in acetone for 10 minutes before drying at room temperature for 20 minutes. The region of interest was marked with a hydrophobic ReadyProbe Barrier Pap Pen (Thermo Fisher Scientific) and left to dry for 60 seconds at room temperature. Samples were promptly blocked with 2.5% BSA in PBS for 30 minutes at room temperature in a hydration tray. Slides were washed three times with PBS for 3 minutes at room temperature. To detect hypertrophy, fluorescently labelled wheat germ agglutinin (WGA, 1:400 W32464, Thermo Fisher Scientific) at the 555 wavelengths was used to detect cellular membranes. Samples were incubated with WGA diluted 1:400 in PBS at room temperature for 30 minutes in a light-proof hydration tray. After this, samples were washed three times with PBS for 3 minutes, as detailed above and stained with NucBlue Fixed cell ready probes reagent (Life Technologies, USA) for 15 minutes at room temperature. Slides were washed as detailed above and mounted with a two drops of fluorescent mount liquid (Fluoromount-G, Thermo Fisher Scientific). Samples were allowed to dry for 30 minutes and then stored at 4°C. Images were acquired on an AxioImagerM1 microscope for fluorescent images. All histological assessments were done manually and completed using Fiji image analysis software (open-source project ImageJ2). In total ten images were randomly captured from around the heart at 40x magnification. Only transverse cardiomyocytes were included in the analysis, while longitudinal cardiomyocytes were excluded to avoid potential variations size due to image position. In general, cardiomyocytes in the sub-endocardial wall are generally orientated longitudinally when imaged in the transverse plane. In total, 15 cardiomyocytes from each image were traced to measure their width and area. The size and average for each image were calculated, and the results from all images were combined to provide an overall assessment of cardiomyocyte size for each mouse.

Molecular biology

Gene expression

Detecting transcripts that drive functional changes in tissue is essential for identifying pathways that may contribute to disease. Applying transcriptome-informed drug design may help better inform candidate selection in drug discovery. This study quantified gene expression changes to evaluate treatment-dependent alterations in pathways known to influence the inflammatory response. Gene expression changes in heart tissue were quantified using qPCR, and a detailed list of primers used in these experiments is provided in the relevant chapter.

Primer design

Primers targeting the genes of interest were designed on Primer-BLAST, utilising the FASTA DNA template. The desired amplicon size was set to between 70- 200 base pairs. Mouse transcripts were selected as the species of interest. Primers were selected based on an optimal annealing temperature of 60°C and a GC% content of 50%. Primers that were suggested to be highly self-complementary were not designed.

RNA extraction

RNA was isolated from heart tissue, with the LV segmented into 30 mg pieces on ice. TRIzol (Thermo Fisher Scientific) was added to the microcentrifuge tube, and the tissue was homogenised with tungsten beads on an electrical tissue lyser at maximum speed for 30 seconds. The sample was removed and placed on ice between homogenisation steps to prevent overheating. Once homogenised, the material was transferred to a new microcentrifuge tube (Eppendorf) without the tungsten beads and incubated at room temperature for 5 minutes to achieve complete nucleotide dissociation. After this, the sample was placed on ice, and 0.2 mL of chloroform was added to the microcentrifuge tube (Eppendorf) and vigorously vortexed at room temperature for 15 seconds. The sample was incubated at room temperature for 3 minutes to achieve aqueous-organic phase separation. The sample was centrifuged for 15 minutes at 4°C at 12,000 xg to complete the phase separation and achieve better RNA purity. The upper phase, identified as the aqueous phase containing the RNA, was removed and aliquoted into a clean microcentrifuge tube. The remainder that contained the RNA and protein was discarded.

To precipitate RNA, 100% isopropanol (Sigma-Aldrich) was added to the microcentrifuge tube (Eppendorf) containing the sample, which was kept on ice. The sample was incubated first at -20°C overnight, followed by a 10-minute incubation at room temperature the next day. RNA was pelleted by centrifugation at 7,500 xg for 10 minutes at 4°C. In the case where RNA concentration was low and the pellet was not visible, the supernatant was carefully removed, and the RNA was resuspended in 75% ethanol (Sigma-Aldrich) on ice. The 75% ethanol removed any remaining salt residual from the nucleic acids. The RNA was then centrifuged a second time at 4°C for 10 minutes at 7,500 xg to pellet the RNA. After this wash, the supernatant was discarded, and the microcentrifuge tube (Eppendorf) was inverted and air-dried for 10 minutes at room temperature to eliminate any remaining ethanol contamination. The RNA pellet was resuspended in 30 μ L of distilled, RNase- and DNase-free water and boiled at 55°C for 10 minutes to reduce RNase activity. After this, the concentration and purity of RNA was measured using a spectrophotometer (Thermo Fisher Scientific, USA). The nanophotometer assessed the 260 nm and 280 nm wavelengths, with the 260/280 ratio of 2.0 indicated pure RNA, with anything lower than 1.8 were excluded. Purified RNA was stored at -80°C until use.

Reverse transcription

The quantification of gene expression with PCR requires the conversion of the RNA template into complementary DNA. This is achieved with an RNA-dependent polymerase, reverse transcriptase that produces single-stranded cDNA copies. A total of 500 ng of RNA was transcribed into complementary DNA with the superscript reverse transcriptase master mix (Thermo Fisher Scientific). Genomic DNA contamination was treated in an initial clean-up step, where RNA and ezDNAase enzyme (Thermo Fisher Scientific) was incubated at 37°C for 5 minutes with purified RNA. This enzyme cleaves the phosphodiester bonds of double-stranded DNA, to eliminate potential genomic DNA inference in downstream applications. After this, the purified RNA was resuspended in a 1:4 ratio with the reverse transcriptase on ice before being transferred to a conventional PCR

for up to 38 cycles. Transcription into cDNA was attained as reported in the instruction manual for superscript IV reverse transcriptase (Thermo Fisher Scientific).

qPCR

Real-time PCR detects the amplification of a cDNA sequence from an initial template to quantify the concentration of that fragment in the reaction. It is a relatively simple approach to measuring gene expression changes, but it is limited to the total number of samples that can fit in a 384-well format. In this thesis, cDNA was resuspended at a concentration of 5 ng per μL . The final concentration of the starting material was determined to be 0.5 ng per well. The amplification of cDNA is completed in the presence of a free-floating fluorescent probe that binds double-stranded DNA. (99) In this case, Sybr Green Master Mix (Thermo Fisher Scientific) was used given its significant cost efficiency and the relative ease at which primers can be designed. This fluorescence signal was quantified on a QuantStudio 12K Flex real-time PCR system (Thermo Fisher Scientific). Data was exported as a CSV file. Triplicates from the run were averaged. Any CT values further than 0.5 CT away from each other were assessed for the accurate CT score based on other replicates. Potential variations in the starting cDNA concentration were normalised by adjusting the CT value from the CT value of the gene of interest to the starting concentration of the material. Housekeeping genes 18s ribosomal RNA (18s) and Actin Beta (Actb) were as reference genes due to their relative abundance. The CT values from the housekeeping genes were removed from the gene of interest to derive a normalised CT value. From here, the normalised CT value from a single mouse in the control group was used to subtract from all other CT values to derive the delta-delta CT. This provides the relative expression of the normalised CT compared to a reference mouse. The $2^{\Delta\Delta\text{CT}}$ was calculated to achieve the fold change. All calculations are presented as a logarithm base 2 and represent the change in gene expression associated with a single transcript between groups.

Protein Expression

Protein expression from the left ventricle of the mouse heart sample was evaluated from the same heart samples from which RNA was isolated. In short, the left ventricle was removed from the -80°C and placed into a new microcentrifuge tube (Eppendorf) on ice. Approximately 200 μL of 1% NP40 buffer (Thermo Fisher Scientific) reconstituted with a protease inhibitor cocktail and phosphatase inhibitor was added, and the sample was homogenised for 3 minutes off and on and placed on ice intermittently to prevent sample degradation. Samples were centrifuged at 10,000 $\times g$ for 10 minutes at 4°C to remove the non-digestible fibrotic components of the tissue. Samples were run on a Pierce bicinchoninic acid assay (Thermo Fisher Scientific) for protein quantification. A standard curve was included to interpolate the concentrations depending on absorbances to acquire a numerical concentration in micrograms per millilitre. Samples were reconstituted at a final concentration of 30 μg for in 40 μL . Samples were reconstituted with 4 μL reducing agent (Thermo Fisher Scientific) and 10 μL loading buffer (Thermo Fisher Scientific) and denatured at 75°C for 10 minutes before being transferred to ice. Proteins were loaded on a 4-12% NuPAGE Bis-Tris protein gel (Thermo Fisher Scientific) and transferred in 1X 2-N-morpholino diluted in deionised water at 120 V for 2 hours (Thermo Fisher Scientific). After

electrophoresis, proteins were transferred from the gel to a polyvinylidene fluoride membrane in transfer buffer (Tri-buffered saline 7.4 pH reconstituted with 20% methanol and 0.1% Tween 20 (Thermo Fisher Scientific)). Membranes were blocked for 1 hour at room temperature in PBS Intercept Blocking Buffer diluted 1:1 with PBS (LI-COR Biosciences, USA). Membranes were then incubated with 1:200 Rabbit polyclonal anti-mP2X7R (Proteintech, USA) and 1:1000 Mouse anti- β -actin antibody (Cell Signaling Technology, USA) overnight at 4°C. The membrane was washed three times for 5 minutes each in TBS with 0.1% Tween 20 and incubated with 1:10,000 secondary fluorescent antibodies targeted against the host in a PBS. The membrane was washed three times with TBS with Tween 20 for 5 minutes. A fourth wash was completed in TBS alone before being imaged on a ChemiDoc MP Imaging System (Bio-Rad Laboratories, USA). The quantification of protein expression was measured based on pixel intensity and expressed at the relative value of the corresponding housekeeper.

Statistical analysis

Each chapter includes a detailed statistical method. Concentration-response curves were presented as a nonlinear, four-parameter fit. Analysis between human cohorts was presented as the mean $\pm$ SEM. Immunophenotyping was evaluated with a multivariate regression analysis. For animal studies, the results were represented as the mean $\pm$ and the standard deviation. Unpaired student T-tests were performed to compare the two groups, and the Gaussian distribution was assumed to be expected between the cohorts. For groups of more than two, a one-way or two-way ANOVA with post-hoc multiple comparisons was used.

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Chapter 3

The P2X7R as a therapeutic target in human coronary artery disease

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Abstract

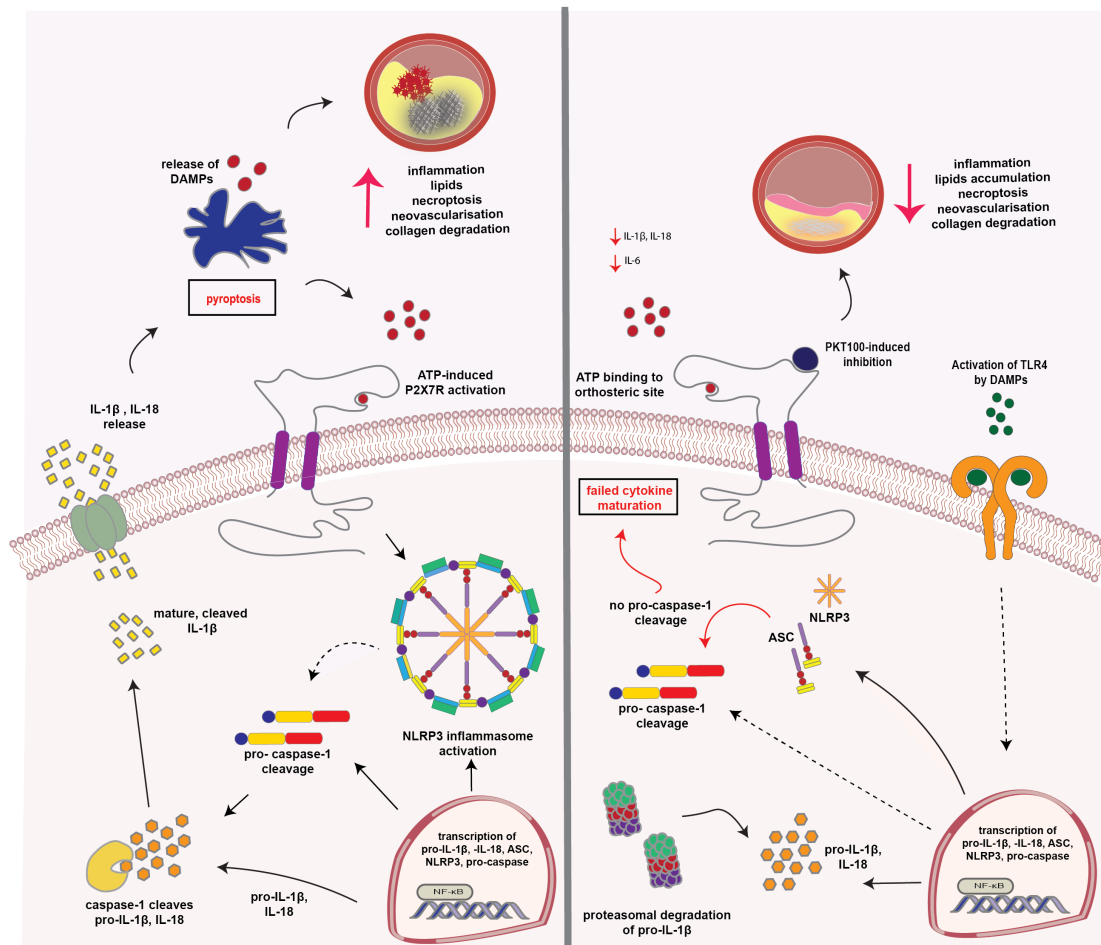
Aims: Despite the well-established link between inflammation and coronary artery disease (CAD), the development of reliable diagnostic tools and effective therapeutic strategies remains a significant challenge. The P2X purinoceptor 7 (P2X7R) is a promising candidate due to its role in the downstream modulation of interleukin-1 beta (IL-1 β) via the NOD- LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome. Herein, P2X7R expression and activity is evaluated in patients with CAD and ST-elevation myocardial infarction (STEMI) and examine the therapeutic potential of a selective P2X7R antagonist, PKT100, to reduce inflammasome activation in patient-derived monocytes.

Methods and results:

Using cytometry by time-of-flight (CyTOF), enrichment of P2X7R expression was observed in classical and non-classical monocytes, CD141⁺ and CD1c⁺ myeloid dendritic cells (mDCs), and a lower proportion of plasmacytoid dendritic cells (pDCs) and natural killer (NK) cells and B cells across cohorts with CAD and STEMI. Notably, heightened P2X7R intensity on non-classical and classical monocytes, as well as CD1c⁺ mDCs, strongly correlated with calcium (CACS) and soft plaque scores (SPS), independent of covariates. In contrast, patients with STEMI exhibited a higher proportion of B cells expressing the P2X7R compared to those without CAD. Equivalent intensity analysis in the STEMI cohort demonstrated reduced P2X7R expression across CD1c⁺ mDCs and classical monocytes that closely correlated with increasing concentrations of C-reactive protein (CRP). Ex vivo evaluation of monocytes enriched from PBMCs through plastic adhesion showed heightened P2X7R-dependent IL-1 β release after 2'(3')-O-(4-Benzoylbenzoyl) adenosine 5'-triphosphate (BzATP) stimulation in participants with CAD. This response was diminished to near baseline concentrations after treatment with a novel P2X7R antagonist, PKT100. The equivalent study was completed in the STEMI cohort. STEMI participants demonstrated heightened IL-1 β release after BzATP stimulation, which could be selectively inhibition after concomitant treatment with PKT100.

Conclusion: BzATP-induced IL-1 β release is heightened in patients with CAD and STEMI compared to no CAD participants, pointing to a potential diagnostic utility. This response was markedly reduced after treatment with P2X7R antagonist PKT100. P2X7R expression was downregulated in classical monocytes from patients with STEMI and showed an inverse correlation with CRP concentrations. Comparatively, P2X7R expression in patients with CAD was positively associated with CACS, suggesting a potential role for P2X7R in the pathophysiology of CAD.

Graphical Abstract



Introduction

Cardiovascular disease (CVD) remains the leading cause of global mortality despite the availability of pharmacotherapeutics to treat modifiable cardiovascular risk. (1,2) Outside of these traditional risks, the role of inflammation is now recognised as a central mediator in atherosclerotic plaque progression and rupture. (3) Particularly, the causal role of cytokine interleukin-1 beta (IL-1 β) in atherosclerosis was long theorised before the clinical benefit was determined. (3,4) In a cohort with a recent history of myocardial infarction (MI) and residual inflammatory risk denoted by elevated C-reactive protein (CRP) concentrations, participants allocated to Canakinumab, an IL-1 β monoclonal antibody demonstrated a 15% reduction in primary outcomes compared to their placebo counterparts. (3) Since then, several anti-inflammatories have followed, achieving various levels of success. (5–7) Despite this, translation of these compounds has been challenging due to the relatively small effect size achieved in studies and a higher rate of fatal sepsis in treatment groups. However, results from these trials have reaffirmed the causal role of inflammation and its importance as a therapeutic target beyond traditional risk factors. More recent attempts to target inflammation in atherosclerosis have moved towards selective approaches that minimise immunosuppression and susceptibility to sepsis. (8–10) Under these circumstances, a substantial unmet need remains in the development of compounds that can safely target the inflammatory contribution to atherosclerosis.

The P2X purinoceptor 7 (P2X7R) is a target of interest in atherosclerosis due to its role as an upstream modulator of IL-1 β . (11) The activation of the receptor is mediated by extracellular adenosine 5'-triphosphate (ATP), released under apoptotic conditions. (12,13) Activation of the P2X7R initiates caspase-1-dependent maturation of precursor cytokines and activation of pyroptotic pathways. (14) These pathways have been demonstrated to be important in the progression of atherosclerotic lesions and might represent a safer way to target inflammation. Several studies have evaluated the P2X7R as a therapeutic target in atherosclerosis. (15,16) Namely, earlier murine models in P2X7R^{-/-} mice demonstrate attenuation of atherosclerotic plaque associated with a lower inflammatory burden. (15,16) In human atherosclerotic lesions, the expression of P2X7R at the gene and protein level has been found in plaques with an unstable phenotype. (17) Thus, while a substantial biological mandate exists to support the clinical evaluation of a P2X7R antagonist in human cardiovascular outcome trials, interest in P2X7R antagonists has been hindered by their limited effectiveness in non-cardiovascular indications. While this may be in partly related to the pharmacological profile of individual compounds, an additional challenge has been detecting clinical benefits “on top of” the anti-inflammatory treatments these patients are receiving as part of guideline-based treatment for their conditions. (12)

The initial adamantane amide P2X7R antagonists, demonstrated strong selectivity and potency at the P2X7R but suffered from a poor half-life. (18) To overcome these deficiencies, bioisosteric substitutions on the adamantane cage was applied, leading to the identification of PKT100 as a promising therapeutic target. (19) Subsequently, systemic administration of PKT100 was demonstrated to reduce adverse right ventricular remodelling and

improve survival in a mouse model of pulmonary hypertension, independent of improvements in pulmonary pressure. (20)

In this present paper, P2X7R expression is explored across peripheral blood mononuclear cells (PBMCs) using cytometry by time-of-flight (CyTOF) in independent cohorts with coronary artery disease (CAD) and ST-elevated myocardial infarction (STEMI). Intensity of the P2X7R was upregulated classical and non-classical monocytes and CD1c⁺ myeloid dendritic cells (mDCs) and correlated with disease burden in patients with CAD. Compartmentally, P2X7R intensity in the STEMI cohort was downregulated in CD1c⁺ mDCs and classical monocyte and negatively correlated with CRP concentration. The release of IL-1 β was assessed in monocyte-derived PBMCs from a sub-cohort of patients with CAD and STEMI. Stimulation with 2'(3')-O-(4-Benzoylbenzoyl) adenosine 5'-triphosphate (BzATP) increased IL-1 β secretion in the supernatants from CAD and STEMI patients. Treatment with PKT100 reduced BzATP-induced IL-1 β release across cohorts. These findings suggest P2X7R as a potential novel therapeutic approach in CAD.

Methods

Study cohort mass cytometry analysis

To characterise P2X7R expression in immune populations, **cohort one** was assembled from participants recruited for suspected coronary artery disease. Clinical data for this cohorts, including comparisons between CAD and no CAD groups, have been already published. (21) Participants were recruited through the BioHEART clinical trial, which was approved by the Northern Sydney Local Health District Human Research Ethics Committee (ACTRN12618001322224). Participant selection for immunophenotyping was based on the availability of PBMCs from the BioHEART-CT discovery cohort, with only samples containing $\geq 1 \times 10^6$ cells included. A total of 15 samples were excluded due to reported technical issues of the experiment, insufficient cell numbers or viability, and abnormal hematological conditions. In total, 117 participants were included in the initial study. (21)

Cohort two was assembled from participants recruited for emergency for percutaneous coronary intervention.

Supplementary Table 1 reports the clinical measurements recorded for groups with STEMI compared to patients without CAD. In total, 92 participants were included in this study. Due to the pragmatic nature of the study, embedded in clinical practice, acute-phase protein measures, including CRP and troponin concentration, were available for STEMI participants, but not for the stable CAD cohort.

CACS and Computed Tomography Coronary Angiogram (CTCA) analysis

Coronary artery calcium score (CACS) and calcium centile were performed using standardised commercial algorithms. For the purposes of analysis, coronary artery segments were divided into 17 segments according to the American Heart Association classification. (20) The degree of coronary stenosis was recorded for each segment, and the Gensini score was calculated. If more than one plaque was present in an individual segment,

only the most stenotic plaque was characterised and recorded. (21) Studies with chronic total occlusions, poor arterial delineation due to motion artefact, or significant blooming artefact due to in situ coronary stents were excluded.

Exploration of specific P2X7R positive immune cell distribution using CyTOF

The methods for staining PBMC populations in cohorts **one** and **two** have been described in previous publications. (21) In short, cryopreserved PBMCs stored in vapour phase in the liquid nitrogen were thawed and washed with RPMI-1640 (Thermo Fisher Scientific, USA) supplemented with 10% heat activated fetal calf serum, 2 mM L-glutamine, 25 μ M 2-mercapto-ethanol, and 1000 units/L of penicillin/streptomycin (Invitrogen, USA). PBMCs were stained for CyTOF analyses, with 41 metal-tagged antibodies listed in Kott et al. 2023. (21) PBMCs were stained with 1.25 μ M cisplatin in phosphate buffered saline (PBS, Thermo Fisher Scientific) for 3 minutes at room temperature and quenched with FACS buffer (eBioscience, USA). Cells were incubated for 30 minutes at 4°C, firstly with anti-CD45 antibodies, in order to facilitate reference sample barcoding with each sample. (22) Following washing and combining accordingly, samples were stained with a cocktail of metal-conjugated antibodies targeting surface antigens. All antibodies, except the fluorescently labelled or metal-tagged antibodies from Fluidigm, were conjugated with MaxPar X8 labelling reagent kits (Fluidigm, USA) according to the manufacturer's instructions by the Ramaciotti Facility for Human Systems Biology, Australia. Following washing with FACS buffer, cells were fixed and permeabilised using eBiosciences FoxP3 buffer kit (USA) at 4°C for 45 minutes and stained with intracellular antibodies for 30 minutes on ice. Following wash steps, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich, USA) containing DNA intercalator (0.125 μ M Iridium-191/193; Fluidigm). After three washes with FACS buffer (eBioscience), cells were filtered through a 35 μ m nylon mesh (Thermo Fisher Scientific) and resuspended to 8×10^5 cells/mL in MilliQ water with EQ beads (Fluidigm) diluted 1 in 10. The samples were then loaded onto the Helios mass cytometer (Fluidigm) and prepared for single-cell flow analysis. Instrument settings were optimised to achieve the maximum number of cells for analysis. Cells were normalised for the signal intensity of EQ beads using the Helios software. FlowJo X 10.0.7r2 software (FlowJo, USA) was used to gate populations of interest, export median signal intensity for markers across populations, and study participants for samples with a minimum of 1×10^4 live cells to analyse. The gating strategy for each of these populations has been described in previous publications. (21)

Study cohort PBMC stimulation

In **Cohort three**, 31 participants who underwent CTCA were recruited for suspected CAD. The study protocol has been previously described in detail elsewhere. (19) Participants were allocated based on their disease status, where 20 participants with CT-defined CAD (CACS > 0 Agatston units (AU)) were defined as the CAD cohort. In contrast, 11 participants were considered to have no CAD (CACS = 0 AU). Demographic and clinical features such as hyperlipidemia, hypertension, and smoking status were based on self-report. Serological markers such as troponin and CRP were measured at a later stage from stored EDTA plasma. Cardiac parameters were not reported for these participants. This cohort excluded patients with previous cardiovascular events, stents, and

bypass operations. Clinical and demographic information was extrapolated from a clinical database after informed written consent was obtained. For some variables, data was not collected and was left blank. **Supplementary Table 2** reports the clinical measures collected.

Cohort four consisted of participants who were either undergoing CTCA for suspected CAD (n=7) or percutaneous coronary intervention (n=17). Due to the emergency associated with their hospitalisation, blood was collected from the arterial sheath following the completion of the procedure. Given the nature of the clinical assessment, biochemical measures such as CRP and peak troponin were measured on admission. Under these circumstances, some participants did not have reported values for left ventricular ejection fraction, CRP or troponin. The details of this cohort are reported in **Supplementary Table 3**. Cardiac systolic measures were reported as ejection fraction from transthoracic echocardiogram. Clinical and demographic information was extrapolated from a clinical database after informed written consent was obtained. For some variables, data was not collected and was left blank.

Preparation of patient-derived monocytes and ex vivo screening

Blood was collected from peripheral venepuncture in the BioHEART-CT cohort and the arterial sheath for the BioHEART-MI population into a lithium heparin-coated tube (Greiner Bio-One, Austria). PBMCs were isolated via density gradient separation as previously described. (21) In short, whole blood was diluted 1:1 with HBSS supplemented with magnesium and calcium (Thermo Fisher Scientific) and transferred to a falcon on top of Ficoll Paque PLUS, density 1.077 g/mL (Cytiva, USA). Samples underwent centrifugation at 770 xg for 15 minutes at room temperature with the brake off. PBMCs were isolated from the interphase area and washed with 14 mL of HBSS before centrifugation at 770 xg for 10 minutes at room temperature with the brake on low. PBMCs were washed once more with 10 mL of HBSS and resuspended in RPMI-1640 cell culture media and seeded at a density of 1.6×10^5 cells/well on a 48-well CellBIND clear bottom plate for 3-hours (Corning, USA) at 37°C to achieve monocyte adhesion. The supernatant was removed, which removed non-monocytic populations, and the remaining monocytes were pre-treated with either 0.01% dimethyl sulfoxide (DMSO) or 1 μ M PKT100 (University of Sydney, Australia) resuspended in RPMI-1640 for 30 minutes at 37°C. Pre-treatments were removed, and cells were washed with warm PBS without magnesium and calcium. Monocytes were split into three conditions: incubated with either (a) 0.01% DMSO in PBS (basal, unstimulated), (b) stimulated with 100 μ M BzATP (MedChemExpress, USA), or (c) 1 μ M PKT100 (School of Chemistry, University of Sydney) in PBS and 0.01% DMSO in combination with 100 μ M BzATP for 30 minutes at 37°C. Supernatants were collected into microcentrifuge tubes and centrifuged at 500 xg for 5 minutes at room temperature. The supernatants were purified by removing supernatants, leaving behind the final 20 μ L behind to ensure no cells were transferred. These samples were stored at -80°C for downstream analysis. The concentration selected was aimed to achieve receptor saturation for ex vivo experiments and was set at ten times the IL-1 β half-maximal inhibitory value (IC₅₀) determined from previous publications. (19)

Detection of pro-inflammatory cytokines from the supernatant and lysates of PBMC-derived monocytes

IL-1 β concentration was measured by a Human Quantikine colourimetric enzyme-linked immunosorbent assay (R&D Systems, USA) from supernatants and lysates from patient monocytes. Immunosorbent plates were pre-coated, to enable validation between batches. Samples were diluted 1:3 in commercial diluent, assayed according to the manufacturer instructions, and absorbance detected at 450/540 nm using a Varioskan LUX microplate reader (Thermo Fisher Scientific).

For pro-IL-1 β experiments, lysate from monocytes was used. Detection of pro-IL-1 β was measured with a Human Pro-IL-1 β Quantikine colourimetric enzyme-linked immunosorbent assay (R&D Systems). Only samples from **Cohort 4** were included in these experiments. Samples were diluted 1:3 in commercial diluent and assayed according to the manufacturer instructions. Absorbance was captured in the 450/540 nm wavelength on a Varioskan LUX microplate reader (Thermo Fisher Scientific).

Caspase-1 activity assay

Following preparation and treatment, adherent monocytes from **Cohort 4** were lysed, and caspase-1 activity was measured using a fluorometric assay kit (Abcam, USA) according to the manufacturer instructions. Output was measured on a Varioskan LUX microplate reader in the 400/505 nm wavelengths (Thermo Fisher Scientific).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 10 (GraphPad Software, USA). To evaluate P2X7R expression and intensity in immune populations in **Cohort one** and **two**, Pearson's correlated to identify initial differences between conditions and assess for covariates. Multivariate regression analysis was performed to assess the association of P2X7R expression (normalised to a reference control sample) on immune cells from **Cohort one**. Clinical parameters such as CACS, SPS and Gensini, which can take infinite values and exhibit zero inflation due to no CAD controls reported as zero, were adjusted using a log (base 10) + 1 transformation to achieve a normal distribution for analysis. Significance was determined by a $p < 0.05$. For the supernatant IL-1 β detection analysis of **Cohort 3** and **4**, a two-way ANOVA followed by multiple comparisons with Tukey's posthoc analysis was performed. Values were presented as the mean $\pm$ standard error mean (SEM) Partial linear regression analysis was used for visualisation purposes. Significant values were considered at $p < 0.05$.

Results

CyTOF reveals P2X7R intensity on a subset of monocytes and CD1c⁺ myeloid dendritic cells (mDCs) that closely correlate with CACS burden.

CyTOF was used to perform comprehensive immunophenotyping on PBMC populations. P2X7R expression across immune subsets from participants with CAD was comparable to those without disease. Irrespective of disease status, P2X7R expression was found to be enriched in classical and non-classical monocytes, CD141⁺ and CD1c⁺ mDCs, as well as a lesser proportion of plasmacytoid dendritic cells (pDCs) and natural killer (NK) cells

(**Figure 1A**). While the proportion of P2X7R-positive cells across subsets did not distinguish patient groups, P2X7R intensity was found to be higher in non-classical ($r^2=0.038$, $p=0.033$) and classical monocytes ($r^2=0.040$, $p=0.030$) and CD1c⁺ mDCs ($r^2=0.055$, $p=0.010$) when compared with CACS burden alone (**Figure 1B**). However, beyond calcification burden, immune cells also modulate their behaviour in response to modifiable risk factors, including hypertension and hypercholesterolemia. (23,24) To address whether P2X7R intensity may be associated with other risk factors contributing to CACS burden, multiple regression analyses was performed, adjusting for covariates. These results showed that P2X7R intensity on classical ($r^2=0.489$, $p=0.035$) and non-classical monocytes ($r^2=0.489$, $p=0.030$) and CD1c⁺ mDCs ($r^2=0.488$, $p=0.035$) retained significance independent of covariates (age, sex, hypertension, hypercholesterolemia, smoking, statin, and anti-hypertensive use) and was associated with CACS burden (**Figure 1C-E**). This demonstrates that upregulation of P2X7R in response to CACS burden may serve as a useful biomarker in the assessment of atherosclerosis progression.

Correlating these measures against other clinical parameters also showed a moderate correlation with SPS in classical ($r^2=0.320$, $p=0.042$) and non-classical monocytes ($r^2=0.240$, $p=0.040$) and CD1c⁺ mDCs ($r^2=0.253$, $p=0.030$) (**Figure 1F-H**). When the same parameters were applied to evaluate P2X7R intensity compared to CACS centile - which denotes susceptibility to CAD, adjusted for sex and age - P2X7R intensity showed a weaker correlation in classical ($r^2=0.040$, $p=0.030$) and non-classical monocytes ($r^2=0.037$, $p=0.036$), CD141⁺ and CD1c⁺ mDCs ($r^2=0.044$, $p=0.022$) (**Figure 1I-J**). The significance of these findings was retained after adjustment for hypercholesterolemia and statin, but not independently of hypertension and anti-hypertensive use. These findings suggest a strong correlation between classical and non-classical monocytes and CD1c⁺ mDCs with CACS burden independent of modifiable risk factors. These distinct immune populations have a crucial role in atherosclerosis development, which underscores the potential utility of P2X7R as a therapeutic and diagnostic target in these cohorts.

CyTOF reveals diminished P2X7R intensity on classical and CD1c⁺ mDCs associated with STEMI.

Next, P2X7R expression patterns across immune cells was examined in patients with STEMI, comparing them to those without CT-defined CAD. The demographic and clinical features of this cohort are reported in **Supplementary Table 1**. P2X7R expression was heightened in classical and non-classical monocytes, CD141⁺ and CD1c⁺ mDCs, and to a lesser extent in pDCs, NK, and B cells (**Figure 2A**). Statistical evaluation with multiple unpaired t-tests revealed a greater proportion of B cells expressing P2X7R in the STEMI group (**STEMI** $2.34\pm1.40\%$, **No CAD** $1.58\pm1.42\%$, $p=0.0124$, **Figure 2B**). To account for significant differences in age and sex within this cohort, these variables were adjusted to evaluate if an increased B cell proportion retained significance. This result persisted in the STEMI cohort after adjustments for age and sex ($p < 0.0001$, **Figure 2C**). Next, P2X7R intensity patterns were explored across immune populations from patients with STEMI compared to those without CAD. Remarkably, CD1c⁺ mDCs ($p=0.044$) and classical monocytes ($p=0.043$) revealed reduced P2X7R intensity in STEMI patients compared to those without CAD (**Figure 2D-F**). Further exploration showed that these features were retained in CD1c⁺ mDCs ($p=0.0072$) and classical monocytes ($p < 0.0001$) after

adjustments for covariates (age, sex, hypertension, type-2 diabetes mellitus, and hypercholesterolemia) (**Figure 2E-F**).

The expression of P2X7R in classical monocytes and CD1c⁺ mDCs prompted an examination of P2X7R intensity in relation to biochemical parameters, peak troponin and hsCRP, which are increased in response to cardiac injury. While there was no association was determined between peak troponin concentrations and P2X7R expression across immune populations, a weak association was revealed in CD1c⁺ mDCs ($r^2 = 0.089$, $p = 0.020$) and classical monocytes ($r^2 = 0.105$, $p = 0.011$) with CRP (**Supplementary Figures 1A-B**). Given the indiscriminate nature of CRP as an indicator of cardiovascular inflammation, covariates known to also affect CRP concentration were adjusted for. Independent of other covariates (age, sex, hypertension, type-2 diabetes mellitus, hypercholesterolemia, and statin use), P2X7R expression in CD1c⁺ mDCs ($r^2 = 0.4$, $p = 0.010$) and classical monocytes ($r^2 = 0.481$, $p = 0.006$) demonstrated a moderate association with CRP (**Figure 2G-H**). These results suggest that P2X7R intensity in classical monocytes and CD1c⁺ mDCs is associated with the heightened inflammatory burden after acute MI. This suggests the potential biological relevance of the receptor in the pathogenesis of atherosclerosis to rupture.

P2X7R dependent IL-1 β release in monocytes from stable patients with CT-defined CAD

In atherosclerosis, the development and progression of lesions are mediated by the immune system. (3,25,26) However, unlike in acute MI, where large-scale hypoxia-induced myocardial injury is associated with the infiltration of immune cells to the myocardium to initiate reparation, atherosclerosis is characterised by persistent low-grade inflammation in the arterial wall. (25,27) P2X7R-mediated IL-1 β from monocytes in patients with well-characterised CAD burden was explored. In total, 31 participants were assessed, with 11 categorised as no CAD compared with 20 participants with CT-defined CAD. A summary of the clinical and demographic characteristics for these participants is presented in **Supplementary Table 2**. Under unstimulated conditions, there were no observable differences in IL-1 β release from monocytes between no CAD and CAD groups (16.9 ± 9.5 pg/mL vs 12.8 ± 3.2 pg/mL, $p = 0.90$, **Figure 3A**). However, a pattern emerged between groups after stimulation with 100 μ M BzATP. Patients with CAD demonstrated 2.08-fold higher BzATP-induced IL-1 β release from monocytes than those without CAD (184.2 ± 33.8 pg/mL vs 88.3 ± 34.1 pg/mL, $p = 0.0039$, **Figure 3A**). This BzATP-induced response was abolished in CAD participants after simultaneous treatment with 1 mM PKT100 (184.2 ± 33.8 pg/mL vs 39.6 ± 11.1 pg/mL $p < 0.0001$, **Figure 3A**). The relative reduction in IL-1 β release from monocytes showed a 78.9 $\pm$ 37.7% decrease after concomitant treatment of PKT100 in the presence of 100 μ M BzATP (**Figure 3B**).

Univariate regression showed a modest association between BzATP-induced IL-1 β release and CACS ($r^2 = 0.16$, $p = 0.024$, **Supplementary Table 4**, **Supplementary Figure 2**). To further elucidate whether the observed difference in BzATP-induced IL-1 β release from PBMCs was associated with CAD burden or risk factors known to contribute to atherosclerosis, multivariate regression modelling was performed, adjusting for covariates

(hypertension, age, sex, and statin use). CRP was also included as a covariate, as IL-1 β has been shown to modulate its expression. (27) After adjustment for covariates that showed no statistical association with BzATP-induced IL-1 β release, the relationship with the quantitative marker of CAD burden- CACS remained significant ($p=0.048$, **Figure 3C**). This indicates that while other covariates account for more of the variance in BzATP-induced IL-1 β release, CACS remains the most significant predictor of its release. This supports existing evidence that P2X7R-dependent inflammation might contribute to atherosclerosis progression. The substantial reduction in BzATP-induced IL-1 β release with PKT100 suggests a potential utility for P2X7R antagonists in the treatment of inflammation in CAD.

Next, intracellular concentrations of IL-1 β at baseline were explored to determine whether they contribute to the heightened extracellular IL-1 β release in response to BzATP in supernatants, reflecting the inflammatory burden of CAD participants. After supernatant experiments, adherent monocytes were lysed, and intracellular IL-1 β concentrations were examined. At baseline, there was no significant difference between CAD participants and those without CAD ($p=0.572$, **Figure 3D**). BzATP stimulatory conditions were investigated to determine its effect on the concentrations of intracellular IL-1 β . There was also no significant difference in intracellular IL-1 β concentrations in response to BzATP ($p=0.929$, **Figure 3D**). Under concomitant treatment with PKT100, there were no observable differences in intracellular IL-1 β . These results indicate that under P2X7R-stimulated conditions, IL-1 β is secreted and returns to baseline concentrations without being stored in the cytoplasm. Furthermore, intracellular IL-1 β does not influence IL-1 β release under BzATP-induced conditions. Overall, these results may reflect a novel opportunity for P2X7R-dependent IL-1 β release to be utilised as a diagnostic indicator of CACS burden and may be of potential use as a companion diagnostic in human trials with anti-inflammatory agents.

P2X7R-dependent IL-1 β release in STEMI participants

The CANTOS trial established the clinical benefit of IL-1 β neutralisation in a cohort with a recent MI. (3) For this reason, the relevance of P2X7R-mediated IL-1 β reduction was assessed in patients presenting with STEMI. In total, 17 patients with confirmed STEMI were compared to 7 participants in whom CTCA had confirmed no CAD. The clinical and demographic characteristics are summarised in **Supplementary Table 3**.

Despite the pathophysiological role of IL-1 β in CAD and MI, it has been challenging to measure IL-1 β in the plasma of patients. This likely reflects its signalling role being localised in areas of inflammation, with activated monocytes deploying their cytokine at a tissue level, as well as its relatively short half-life that is unlikely to reach the circulation (28) Therefore, monocytes isolated from the PBMCs from STEMI participants were evaluated for IL-1 β secretion. Under baseline conditions, there were no observable differences in IL-1 β secretion from STEMI monocytes compared to the no CAD group (22.7 ± 10.4 vs 6.46 ± 1.87 ; $p=0.416$, **Figure 4A**). However, much like PBMCs from CAD patients, treatment with 100 μ M BzATP resulted in a 2.8-fold greater IL-1 β release from STEMI monocytes compared to the no CAD group (70.0 ± 16 pg/mL.4 vs 24.3 ± 15 pg/mL.; $p=0.024$, **Figure 4A**). To explore

whether PKT100 can reduce heightened BzATP-induced IL-1 β from STEMI monocytes, monocytes were concomitantly treated with BzATP and 1 μ M PKT100. Treatment with PKT100 substantially reduced BzATP-induced IL-1 β release in STEMI monocytes to near baseline concentrations (24.6 ± 7.4 vs 70.0 ± 16.4 ; $p=0.010$, **Figure 4A**). Similar to patients with CAD, the relative magnitude reduction in IL-1 β was measured after incubation with PKT100. Concomitant treatment with PKT100 resulted in a $58.3 \pm 26.28\%$ in IL-1 β in the STEMI cohort (**Figure 4B**). This data supports P2X7R antagonism to attenuate IL-1 β dependent inflammation in STEMI. Consistent with the lack of BzATP-induced IL-1 β release from the no CAD group, PKT100 had no significant effect in this cohort.

Human studies have proposed the prognostic value of acute-phase proteins such as CRP after MI. (29) Particularly, CRP concentrations are associated with elevated risk of future adverse cardiovascular events. (3) Since CRP concentration is partially regulated by IL-1 β activity, the association between CRP concentration and IL-1 β in the STEMI groups was explored. Neither baseline nor BzATP-induced concentrations of IL-1 β were correlated with peak CRP concentration (**Figure 4C**). There was also no association between troponin concentrations under baseline conditions or after ATP-induced IL-1 β concentration (**Figure 4D**).

P2X7R-activation mediates intracellular maturation of IL-1 β from its precursor form.

The mechanisms underlying P2X7R-mediated IL-1 β release in STEMI patients was further investigating. Given the monocytes were stimulated with BzATP for only a brief timeframe before the supernatant was collected, it was hypothesised this duration was sufficient to induce maturation and release of IL-1 β from its precursor form but could not facilitate newly synthesised precursor IL-1 β . Under these circumstances, the intracellular concentration of Pro-IL-1 β was measured from monocytes. Pro-IL-1 β was elevated 1.9-fold in STEMI monocytes compared to the no CAD group at baseline (569.1 ± 74.2 vs 294.8 ± 57.2 , $p=0.04$, **Figure 5A**), consistent with heightened activation of pro-inflammatory pathways in the setting of the MI. (3) To evaluate whether BzATP-induced activation of the P2X7R would deplete pro-IL-1 β concentrations as the protein is cleaved into its active form, intracellular concentrations of pro-IL-1 β were measured from monocytes after stimulation. There were no observable differences in pro-IL-1 β after BzATP stimulation in monocytes from STEMI participants compared with unstimulated monocytes from the same cohort (523.8 ± 72.2 vs 569.1 ± 74.2 , $p=0.62$, **Figure 5A**). These results suggest that P2X7R stimulation does not alter the overall concentrations of Pro-IL-1 β , a finding consistent with previous observations, (30) where only a fraction of the existing pooled concentration affected.

Next, it was examined whether intracellular concentrations of mature IL-1 β were modified under P2X7R stimulation with BzATP in STEMI participants. Under baseline conditions, there were no observable differences in intracellular IL-1 β between STEMI and no CAD groups in monocytes (24.6 ± 9.0 vs 7.9 ± 4.0 , $p=0.36$, **Figure 5B**). However, in response to BzATP stimulation, intracellular IL-1 β was 6.6-fold greater in STEMI participants compared to the no CAD group after BzATP stimulation (66.0 ± 24.6 vs 10.0 ± 5.9 , $p=0.0031$, **Figure 5B**). This is largely reflective of an increase in intracellular IL-1 β after BzATP stimulation, although no difference was

detected between baseline and BzATP treatment in the STEMI group (66.0 ± 24.6 vs $24.6.0 \pm 9.9$, $p=0.076$, **Figure 5B**). In response to P2X7R antagonism with PKT100, increases BzATP-induced in intracellular IL-1 β in STEMI participants was not significantly inhibited (66.0 ± 24.6 vs 33.1 ± 15.4 , $p=0.176$, **Figure 5B**). In accordance with the concentrations observed at baseline, the intracellular IL-1 β concentration measured after PKT100 inhibition might reflect the inflammatory status of a patient.

Lastly, the relationship between P2X7R activation and caspase-1 activity was examined. Caspase-1 has long been recognised for its critical role in cytokine maturation and the activation of pyroptotic pathways. (31–34) Precursor IL-1 β is inactive after synthesis and resides in intracellular compartments. (34) It was hypothesised that activation of the P2X7R would promote caspase-1 activity in STEMI PBMCs to facilitate IL-1 β maturation. Stimulation with 100 μ M of BzATP did not significantly increase caspase-1 activity compared to baseline (12.5 ± 2.2 vs 5.9 ± 2.1 , $p=0.0678$, **Figure 5C**). While BzATP-induced caspase-1 activation did not meet predetermined measures of statistical significance, it was examined if PKT100 reduced caspase-1 activity. There was no observable statistical difference in caspase-1 activity in response to PKT100 inhibition (12.5 ± 2.2 vs 7.4 ± 1.9 , $p=0.185$, **Figure 5C**). In line with the above results in **Figures 5A-B**, this data may suggest the extracellular release of BzATP-induced IL-1 β may occur through other pathways such as, pyroptosis that need further exploration.

Discussion

While IL-1 β is well established for its causal role in coronary atherosclerosis, the current data presented suggests improved specificity of P2X7R-dependent IL-1 β release relevant to both diagnosis and risk stratification of CAD. Strong changes in P2X7R intensity were observed across specific immune populations in both CAD and MI, suggesting an intricate role in both chronic inflammation of atherosclerosis and acute inflammation associated with myocardial damage. In particular, P2X7R intensity on CD1c⁺ mDCs and classical and non-classical monocytes was strongly predictive of CACS- a well-validated marker of CAD burden. BzATP-induced IL-1 β release from patient monocytes demonstrated a substantially closer relationship with coronary atherosclerotic burden and plaque destabilisation than unstimulated IL-1 β or plasma IL-1 β . and is abolished by the newly developed P2X7R antagonist, PKT100. This data provides strong evidence for using the P2X7R as a therapeutic target to reduce IL-1 β -dependent pathophysiological signalling in CAD. It also offers fundamental evidence for its application as a novel diagnostic tool for those with suspected CAD.

The high dimensionality advantage of CyTOF has been utilised in recent studies to assess heterogeneity in immune cell phenotypes in human disease states, including cardiovascular disease. (35) The potential of specific immune subpopulation signatures to be used as diagnostic biomarkers is supported by the recent publication identifying higher proportions of T-regulatory cells in patients with CT-defined CAD. (21) This approach moves beyond standard immune cell surface markers required to identify subpopulations to explore differences in the expression of cell-surface receptors implicated in inflammatory signalling pathways. For the first time,

expression of the P2X7R was demonstrated across classical and non-classical monocytes, CD141⁺ and CD1c⁺ mDCs, pDCs, NK and B cells. In a cohort with CAD, stratification based on CT-defined coronary plaque revealed a higher intensity of P2X7R expression among CD1c⁺ mDCs and classical and non-classical monocytes. These significant associations persisted throughout the assessment of other risk matrices, such as SPS, but were not sustained with CACS centile after adjustment. These results may reveal a critical link of P2X7R upregulation in response to atherosclerotic plaque progression and destabilisation.

Intriguingly, in a cohort with STEMI, P2X7R expression was reduced in classical monocytes and CD1c⁺ mDCs compared to those without CAD. The observed reduction in P2X7R expression on classical monocytes was associated with the rising concentrations of CRP in STEMI participants. This phenomenon is unlikely to be attributed to cellular apoptosis as it was demonstrated that the proportion of P2X7R-expressing cells remains consistent. Instead, it appears that in the acute state, the expression of P2X7R is reduced, reflecting potential receptor internalisation. This phenomenon has been previously characterised in vitro, where synthetic agonists are demonstrated to induce membrane internalisation of the receptor. (36,37) Alternatively, P2X7R has been detected in plasma and serum samples and is suggested to shed in response to inflammation. (38) In this setting, heightened concentrations of P2X7R in serum samples were strongly correlated with greater CRP concentrations from individuals after MI. (38)

Monocytes have been extensively studied in the context of atherosclerotic plaque development, with studies underscoring their critical role in the pathogenesis of the disease. (24,25,35,39) While early in the atherogenic process, monocytes are equipped to respond to the increasing lipid and inflammatory demands at vascular walls, as macrophages they are soon exhausted and deposited in atherosclerotic lesions as cellular debris. (39,40) Classical monocytes are distinguished from non-classical monocytes based on their expression of CD14⁺ and CD16⁺ markers. (41) Although both are recruited to vascular walls, classical monocytes take an active role through diversification into macrophages to form foam cells. (24) Despite their relatively short life cycle, recent evidence supports innate memory, whereby leukocytes adopt a memory state in chronic inflammatory conditions. (40,42) P2X7R was abundantly expressed in these populations. Under these circumstances, targeting the P2X7R may represent an important therapeutic strategy against inflammation in atherosclerosis.

By comparison, non-classical monocytes have been labelled as anti-inflammatory due to their reduced capabilities to secrete cytokines and facilitate phagocytic functions. (43) Their localisation to the vasculature underscores their critical contribution to the maintenance or 'patrolling' of vascular walls close to atherosclerotic lesions. (44) The increased proportion of these cells has been previously associated with intima-media thickening, irrespective of increases in other leukocyte populations, including classical monocytes. (44) These populations have also demonstrated greater superoxide production in patients with CAD. (45) In this case, the increased expression of P2X7R in non-classical monocytes reveals a potential role for this population in cytokine secretion, which better aligns with more recent literature. (46,47) CD1c⁺ mDCs have also been recently

associated with plaque destabilisation. (48) Moreover, their critical regulation as a moderator between the innate and adaptive immune systems makes them an essential regulator of atherosclerosis. (49) Under these circumstances, the P2X7R may be vital in this process. These findings point to the potential therapeutic value of the treatment of inflammation associated with atherosclerosis independent of other risk factors through the P2X7R.

Despite the established role of inflammation in atherosclerosis development, progression, and instability, tools in measuring CAD in the silent phase are limited. While available clinically, CRP is limited in specificity and has predominantly demonstrated use at a population level, including for enrichment in clinical trial design. (50) In the CT-defined CAD cohort in this study, CRP levels measured from plasma were no different from those without CAD. Detecting IL-1 β as a diagnostic target has been hindered by a short half-life and low abundance in plasma, partially reflecting the “distance” from focal release at the tissue level, posing significant challenges to its reliable detection in epidemiological studies. (28) The prognostic potential of BzATP-induced IL-1 β release to predict CAD burden points to a possible diagnostic role for personalised CAD detection, risk stratification and heart attack prevention. The dependence of this prognostic ability on stimulation with BzATP, a potent and specific synthetic P2X7R agonist, supports the particular importance of P2X7R-dependent-IL-1 β in CAD. Such a tool requires further evaluation. However, as it is trained against CAD on non-invasive imaging, it has the potential to be applied in primary preventative care pathways to facilitate the triaging of patients for imaging for subclinical disease in a manner akin to faecal occult blood screening for bowel cancer to triage colonoscopy in asymptomatic adults. (51) It also has the potential for enrichment into clinical trials targeting inflammation to improve the power of the study and reduce study size. Currently, studies such as CANTOS have relied on CRP > 2 mg/L but have required 10,000 participants for adequate power. (3)

Finally, this study points to the therapeutic potential of P2X7R antagonism and demonstrates the efficacy of the recently developed antagonist PKT100. The observation that patient-derived PBMCs retained an imprint of their CAD inflammatory state with heightened P2X7R-dependent IL-1 β release enhances the likelihood of clinical relevance of this ex vivo finding. These results suggest that P2X7R antagonism may be an effective therapeutic strategy to inhibit systemic inflammation in patients with CAD and residual risk during secondary prevention treatment after STEMI. The use of P2X7R antagonists in cardiovascular disease research has been limited, focusing largely on other components downstream of NLRP3 inflammasome for atherosclerosis, such as monoclonal antibodies against IL-1 β . (3) In contrast, a number of P2X7R have shown early-stage promise to progress to phase III clinical trials for systemic inflammatory conditions, including inflammatory bowel disease and rheumatoid arthritis AZD9056 or JNJ-54175446. (52,53) Despite this, clinical efficacy against primary endpoints has not been demonstrated for these conditions. This may partly relate to the need to demonstrate benefits above and beyond optimal anti-inflammatory treatment, including steroids, a challenge not faced by new therapies for CAD.

While the CANTOS study demonstrated a reduction in recurrent atherosclerotic events by administering a monoclonal antibody against IL-1 β , it did so at the cost of a small but significant increase in fatal bacterial infections. (3) Furthermore, post hoc analyses of the CANTOS study determined that levels of IL-18 were unaffected by IL-1 β neutralisation, while levels of IL-6 were decreased but remained a significant contributor to future cardiovascular risk. (54) In this instance, P2X7R antagonism could be therapeutically advantageous, given its role in the biological modulation of IL-1 β and IL-18, thereby modulating downstream IL-6 levels with a more comprehensive impact required for CAD. (55) Moreover, the potential advantage of P2X7R antagonism extends to its capabilities as a modulator of 'sterile' inflammation, while microbial components independent of the NLRP3 inflammasome and toll-like receptor-4 were partially preserved (56,57), with likely clinical benefits of lower risk of sepsis compared with monoclonal antibody targeting IL-1 β . The potential benefit of IL-1 β antagonism with P2X7R may also extend to the myocardium and other cardiovascular disease states.

Limitations

There are some limitations to the study. The *ex vivo* monocyte model requires cell culture and BzATP treatment to activate the P2X7R pathway, which may exert phenotypic alterations in monocytes compared to *in vivo* conditions. The novel compound PKT100 is a potent inhibitor of the P2X7R in an *ex vivo* human settings; however, the present study does not assess its *in vivo* efficacy in atherosclerosis, which may significantly differ given the complexities of the atherogenic process. Furthermore, there was no PKT100 treatment group that was independent of BzATP. These findings have set the stage for further *in vivo* investigations of PKT100 in a model of atherosclerosis.

Conclusion

In conclusion, P2X7R-dependent IL-1 β release has been identified as a more specific diagnostic and therapeutic target in coronary atherosclerosis than standard measures of IL-1 β . The expression of P2X7R was found on classical monocytes in STEMI participants, and it showed a close association with CRP concentration. This pathway indicates that reducing P2X7R is a deleterious event in the setting of MI. In cohorts with CAD, P2X7R intensity was increased in non-classical monocytes and CD1c⁺ mDCs, showing moderate correlations with the CACS percentile. These findings point to using P2X7R expression to help screen individuals at risk for future cardiovascular events and the further translational development of novel agents, including PKT100.

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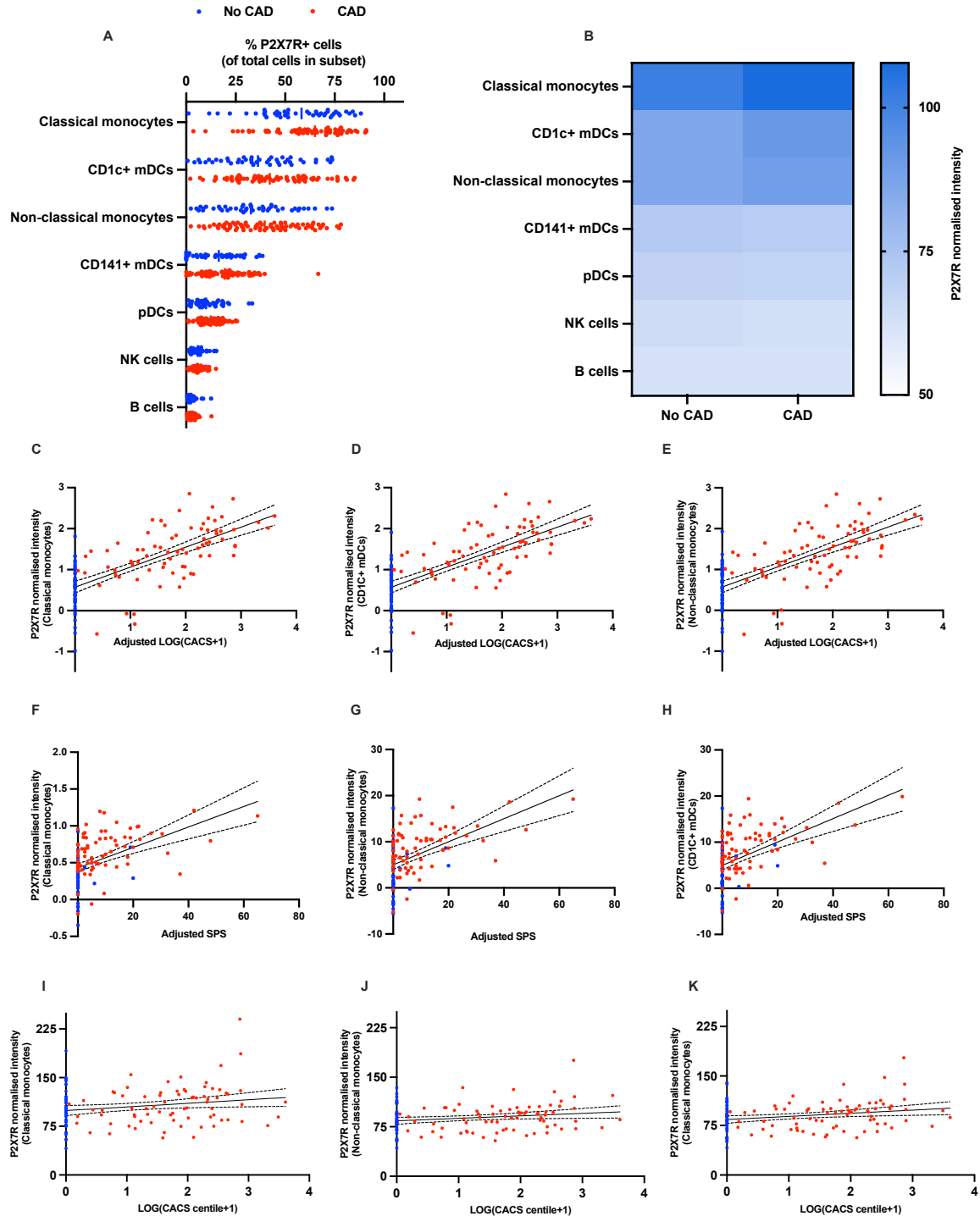


Figure 1. CyTOF reveals heightened expression of P2X7R among non-classical and classical monocytes and CD1c+ mDCs in CAD patients. **A)** Proportion of cells that express the P2X7R. **B)** Heat map illustrating the median P2X7R intensity across different immune population. Partial regressions between adjusted log (CACS+1) and the intensity of P2X7R on **C)** classical monocytes, **D)** non-classical monocytes, and **E)** CD1c+ mDCs. Partial regressions between adjusted SPS and the intensity of P2X7R on **F)** classical monocytes, **G)** non-classical monocytes, and **H)** CD1c+ mDCs. Partial regressions between adjusted log (CACS centile+1) and the intensity of P2X7R on **I)** classical monocytes, **J)** non-classical monocytes, and **K)** CD1c+ mDCs. Partial regressions based on multivariate regression analyses. Data presented as normalised median P2X7R intensity.

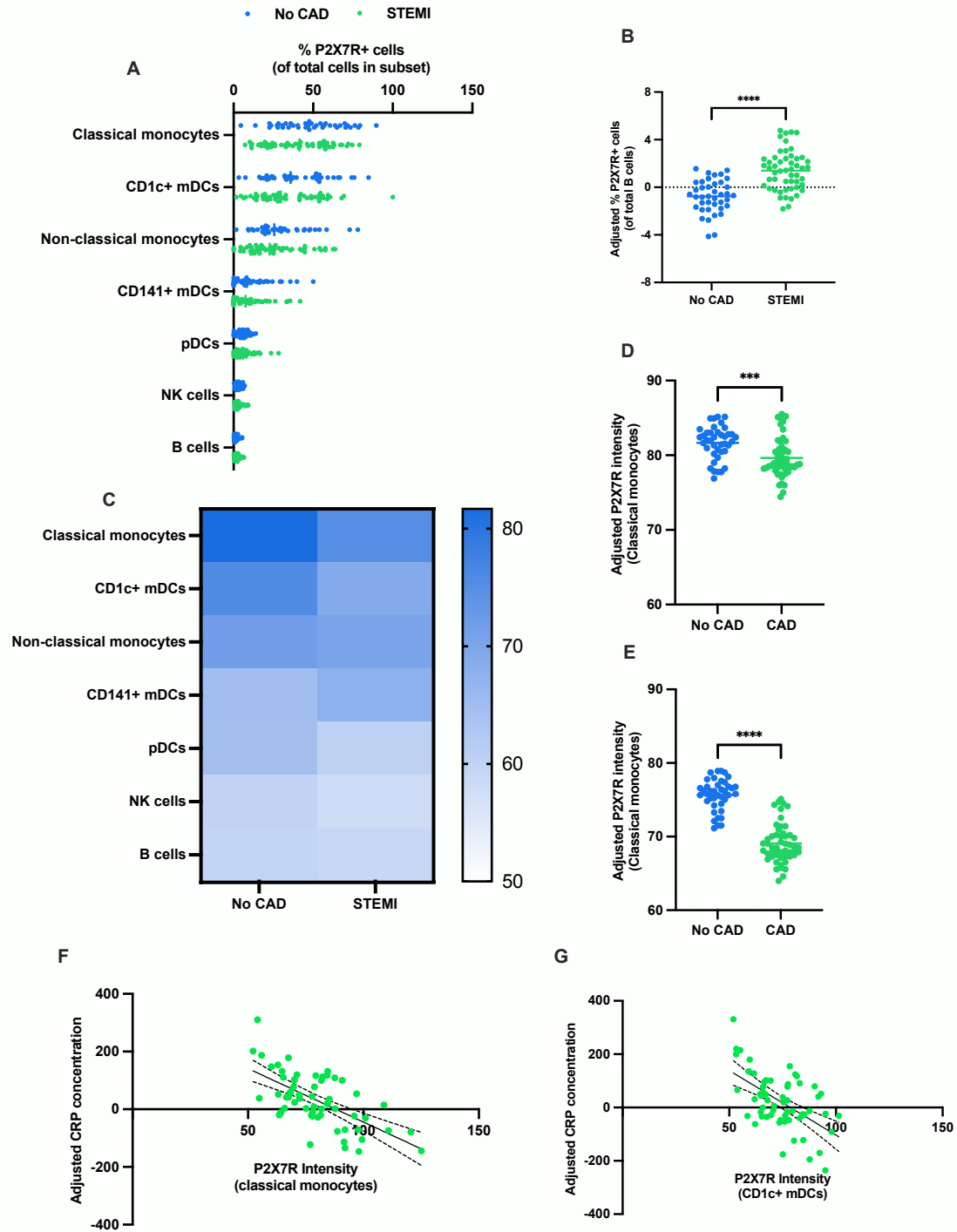


Figure 2. CyTOF reveals lower intensity of P2X7R among classical monocytes and CD1c+ mDCs in STEMI patients. **A)** Proportion of P2X7R expressing cells. **B)** Dot plot showing B cells expressing the P2X7R in no CAD vs STEMI after adjustment. **C)** Representation of the P2X7R across different immune populations in STEMI and no CAD controls. **D)** Dot plot showing P2X7R expression on classical monocytes in no CAD vs STEMI participants, adjustment for sex and age **E)** Dot plot showing P2X7R expression on CD1c+ mDCs in no CAD vs STEMI participants, adjustment for sex and age **F)** Partial regression analysis comparing between adjusted CRP concentration (age, sex, hypertension, and hypercholesterolemia, type-2 diabetes mellitus and statin) and P2X7R intensity in classical monocytes **G)** Partial regression analysis between adjusted CRP concentration (age, sex, hypertension, and hypercholesterolemia, type-2 diabetes mellitus and statin) and P2X7R intensity in CD1c+ mDCs. Data presented as normalised median P2X7R intensity. ****P<0.0001.

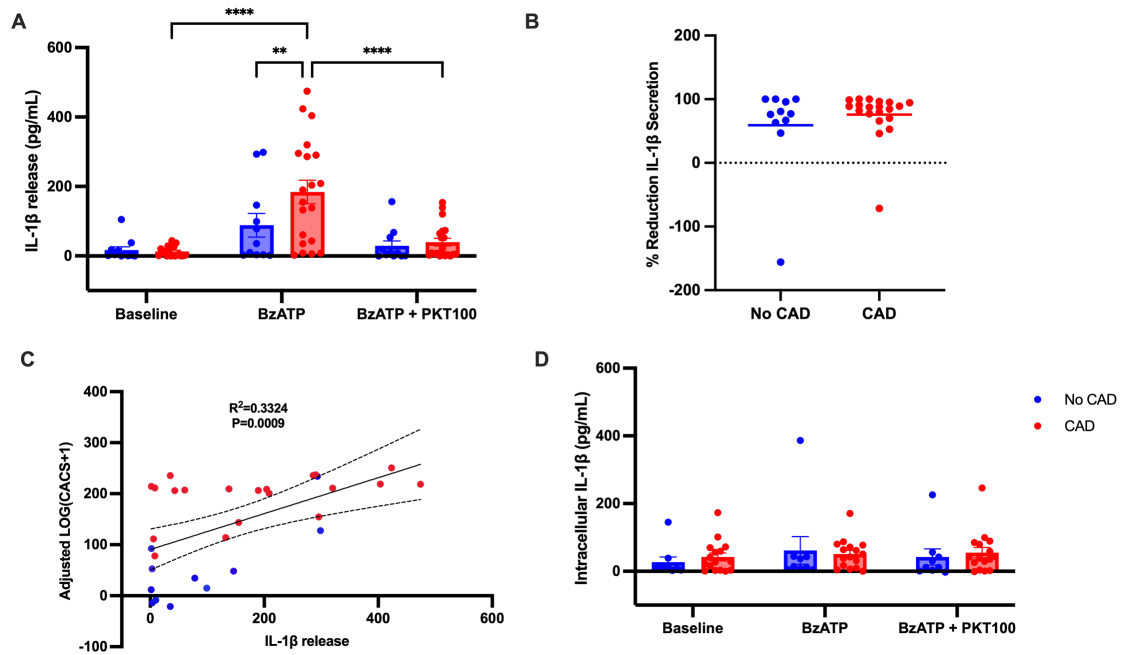


Figure 3. CAD monocytes exhibit significant elevation in IL-1 β in response to ATP stimulation. PKT100 reduces IL-1 β secretion down to near baseline levels. A) Concentrations of IL-1 β secreted by monocytes (no CAD = 11, CAD: 20). **B)** Percentage magnitude reduction in IL-1 β release elicited by PKT100. **C)** Adjusted BzATP-induced IL-1 β release values from monocytes from multiple-regression analysis demonstrate a better correlation with CACS score. **D)** Intracellular concentrations of IL-1 β (No CAD = 11, CAD = 16). Data are presented as mean $\pm$ SEM. ** $P<0.005$, *** $P<0.0005$, **** $P<0.0001$.

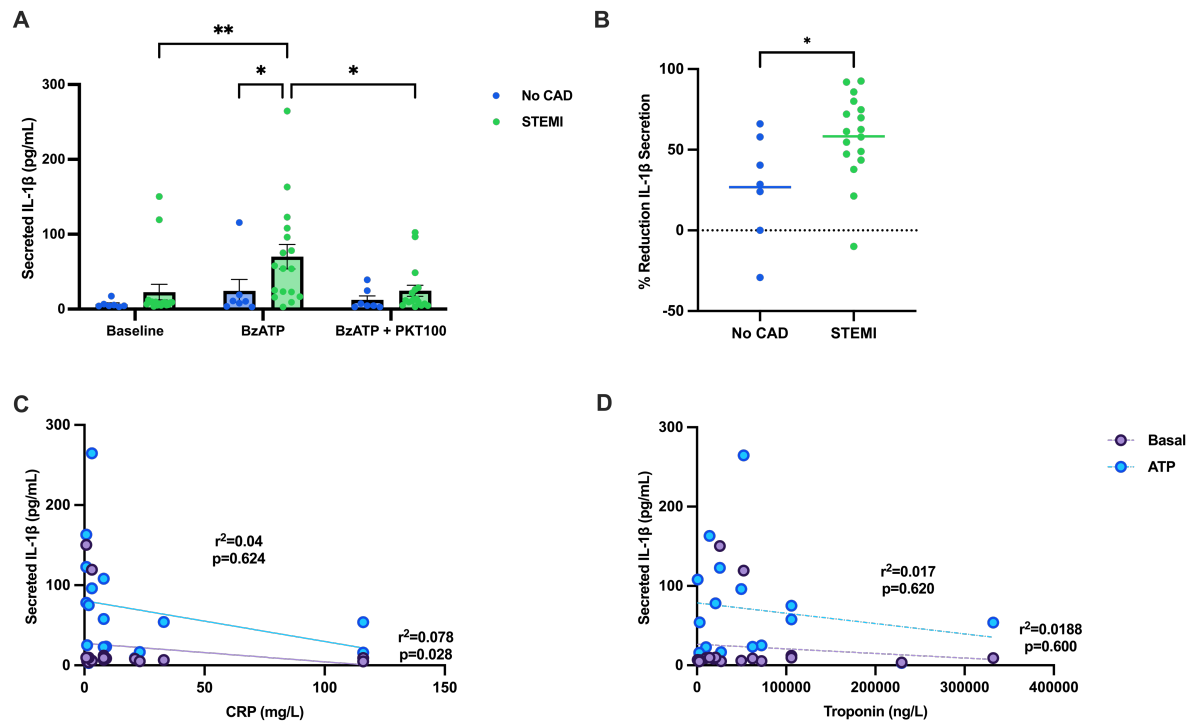


Figure 4. PKT100 potently inhibits IL-1 β in STEMI-derived monocytes. **A)** Bar graphs demonstrating the concentrations of secreted IL-1 β from monocytes present in supernatant from no CAD and STEMI participants following baseline, BzATP-induced and with co-incubation of PKT100 in the presence of BzATP (**No CAD = 7, STEMI = 17**). **B)** Dot plots showing the magnitude of IL-1 β reduction in response to PKT100 treatment in no CAD and STEMI participants (**No CAD = 7, STEMI = 17**). **C)** Correlation between baseline and BzATP-induced levels of IL-1 β with plasma CRP (**N = 17**). **D)** Correlation between baseline and BzATP-induced concentrations of IL-1 β with plasma troponin measured at admission (**N = 17**). Data presented as mean $\pm$ SEM. *P<0.05, **P<0.005.

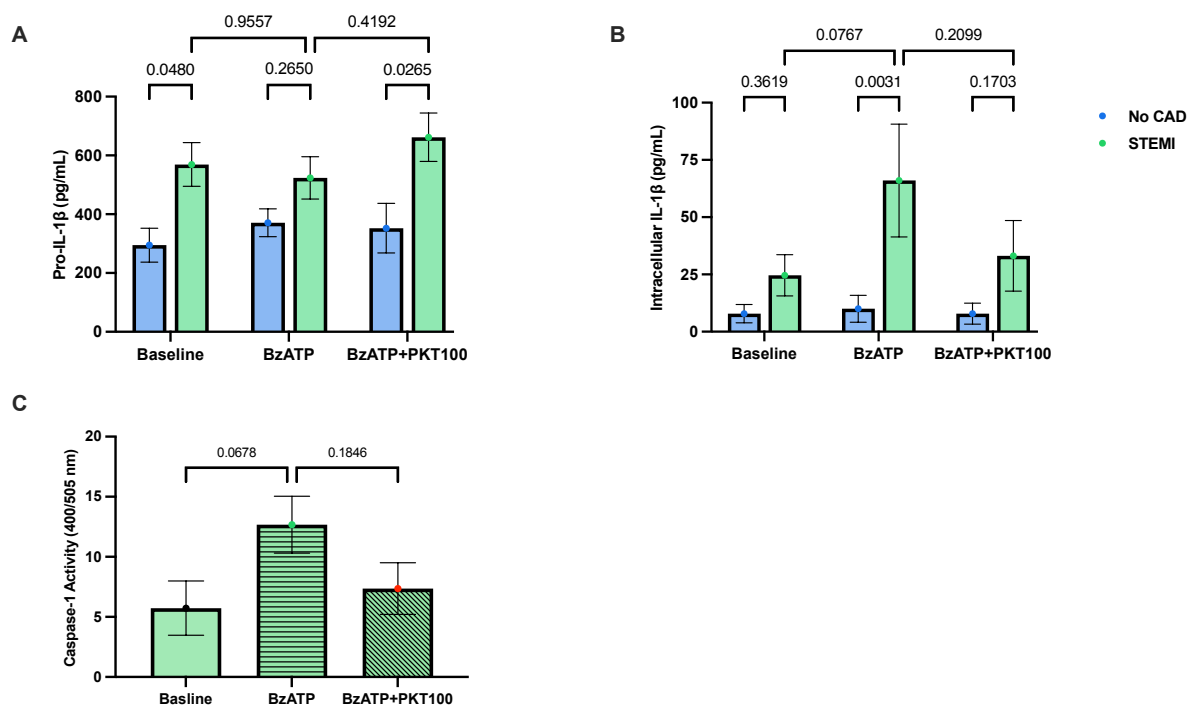


Figure 5. PKT100 antagonism exhibits an anti-inflammatory function via a caspase-1-dependent reduction in pro-IL-1 β cleavage to its mature form in STEMI participants. A) Pro-IL-1 β levels in STEMI and no CAD participants under baseline, BzATP-induced stimulation and concomitant treatment with PKT100 (**No CAD = 5, CAD = 14**). **B)** Concentrations of intracellular IL-1 β in STEMI and no CAD participants under baseline, BzATP-induced stimulation and concomitant treatment with PKT100 (**No CAD = 12, CAD = 12**). **C).** Caspase-1 activity in STEMI participants under baseline, BzATP-induced stimulation and concomitant treatment with PKT100 (**N=12**). Data are presented as mean $\pm$ SEM.

Supplementary Table 1. Key clinical and demographic characteristics of Cohort two used in CYTOF analysis

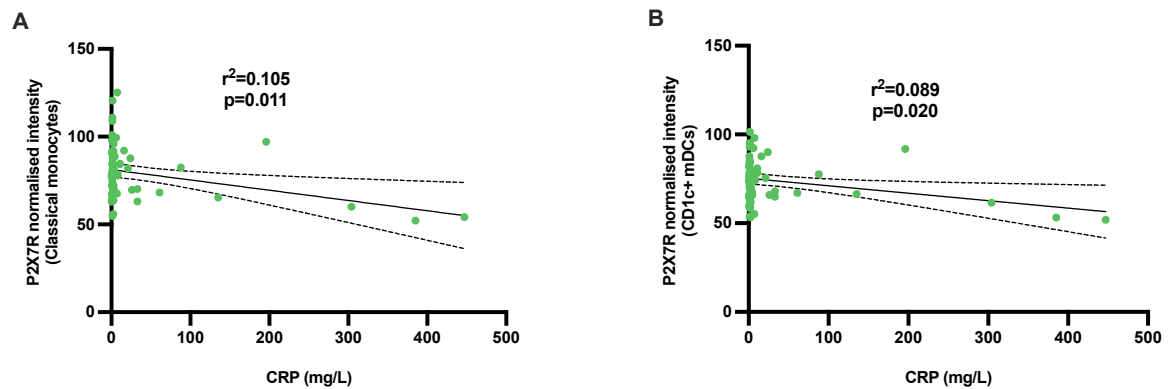
	No CAD (40)	STEMI (52)
CT Calcium Score, γ -median (IQR)	0	-
Age, γ -mean (SD)	53.3 $\pm$ 12.8	63.7 $\pm$ 14.1
Female – no. (%)	22(55.0)	15(28.8)
Hypertension – no. (%)	12(30.0)	21(52.5)
Diabetes – no. (%)	3(7.5)	8(20.0)
Hyperlipidemia – no. (%)	17(42.5)	26(65.0)
Significant smoking history– no. (%)	4(10.0)	18(45.0)
Statin – no. (%)	8(20.0)	13(32.5)
Antithrombotic agent - no. (%)	3(7.5)	6(15.0)
Anti-hypertensives – no. (%)	12(30.0)	14(35.0)
Median high-sensitivity CRP level – mg/L	1.34 $\pm$ 0.78	8.0 $\pm$ 44.1
Median troponin (IQR)	1.10 $\pm$ 1.20	270444 $\pm$ 59740

Supplementary Table 2. Key clinical and demographic characteristics for cohort three used in patient-derived monocyte assay

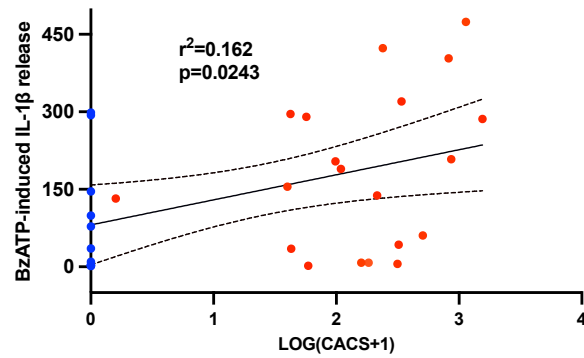
	No CAD (11)	CAD (20)
CT Calcium Score, γ -median (IQR)	0	198.0 $\pm$ 323.0
Age, γ -median (IQR)	59.0 $\pm$ 17.0	72.0 $\pm$ 13.3.0
Female – no. (%)	9(81.8)	10(50.0)
Hypertension – no. (%)	4(36.4)	10(50.0)
Diabetes – no. (%)	2(18.2)	1(5.0)
Hyperlipidemia – no. (%)	3(27.3)	15(75.0)
Significant smoking history– no. (%)	3(27.3)	10(50.0)
Statin – no. (%)	3(27.3)	6(30.0)
Antithrombotic agent - no. (%)	2(18.2)	4(20.0)
Anti-hypertensives – no. (%)	3(27.3)	12(60.0)
Median high-sensitivity CRP level – mg/L	1.69 $\pm$ 1.69	0.910 $\pm$ 1.77
Median troponin (IQR)	1.00 $\pm$ 1.10	2.30 $\pm$ 2.20

Supplementary Table 3. Key clinical and demographic characteristics of cohort four used in patient-derived monocyte assay

	No CAD (7)	STEMI (17)
Age, y-median (IQR)	52.0 ± 25.0	63.0 ± 12.0
Female – no. (%)	3(42.9)	4(23.5)
Hypertension – no. (%)	4(57.1)	9(52.9)
Diabetes – no. (%)	0(0)	4(23.5)
Hypercholesterolemia – no. (%)	2(28.6)	10(58.8)
Significant smoking history– no. (%)	0(0)	6(35.3)
Statin – no. (%)	1(14.3)	6(35.3)
Antithrombotic agent - no. (%)	0(0)	2(11.8)
Anti-hypertensives – no. (%)	4(57.1)	9(52.9)
Median high-sensitivity CRP level – mg/L	1.05 ± 0.680	22.0 ± 45.00
Median troponin (IQR)	1.20 ± 0.800	26959.0 ± 58228.0
Ejection Fraction, y-mean (SD) (%)	-	47.81 ± 9.99



Supplementary Figure 1. Unadjusted P2X7R intensity and its relationship with CRP in the STEMI cohort. A) Shows unadjusted P2X7R intensity on classical monocytes in relation to CRP concentration. **B)** Shows unadjusted P2X7R intensity on CD1c+ mDCs in relation to CRP concentration.



Supplementary Figure 2. Linear relationship between BzATP-induced IL-1 β and LOG (CACS+1)

Supplementary Table 4. Multiple linear regression analysis

Variable	Estimate	SE	95% CI	t	P value
Intercept	184.8	165.0	-155.8 to 525.3	1.120	0.274
Age	-2.831	2.607	-8.2 to 2.548	1.086	0.289
Sex	86.96	58.97	-34.75 to 208.7	1.475	0.153
CACS	58.61	28.09	0.6362 to 116.6	2.087	0.048*
CRP	-3.883	9.284	-23.04 to 15.28	0.4183	0.680
Hypertension	84.55	53.33	-25.53 to 194.6	1.585	0.126
Statin	-10.60	54.32	-1227 to 101.5	0.1952	0.847

^a Multivariate linear regression analysis demonstrating the relationship between BzATP-induced IL-1 β secretion. and traditional risk factors associated with CAD, including sex, age, and hypertension.

^b Multivariate analysis included statins, given the presumed anti-inflammatory benefits.

^c CRP is included as a potential predictor of vascular inflammation in CAD.

Chapter 4

PKT100 exhibits a therapeutic benefit on plaque stabilisation through inhibition of the P2X7R

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Abstract

The success of the CANTOS trial in reducing nonfatal myocardial infarction (MI), nonfatal stroke, and cardiovascular mortality through neutralisation of a single inflammatory cytokine, interleukin-1 beta (IL-1 β), has opened new therapeutic avenues for the treatment of atherosclerosis. Targeting upstream modulators of inflammation is thought to hold a greater therapeutic benefit as it allows for the simultaneous inhibition of multiple molecules, offering broader target selection. Due to its critical role in sterile immunomodulation, the P2X purinoceptor receptor 7 (P2X7R) has been identified as a promising therapeutic target. In a mouse model of atherosclerosis, the ablation of the P2X7R was associated with attenuation of atherosclerotic lesions. Despite this, the rupture of atherosclerotic lesions remains the leading contributor to the global burden of cardiovascular disease. In this study, the efficacy of a novel P2X7R antagonist, PKT100 to stabilize atherosclerotic lesions was explored in a mouse model of plaque instability. 6-9-week-old apolipoprotein E knockout mice received a high-fat diet for 7 weeks, at which point mice underwent tandem right carotid ligation. Mice commenced treatment with either 0.2 mg/kg PKT100 or vehicle for 6-weeks. 6-weeks after postoperative ligation, the carotid bed was removed, and plaques were assessed for features of plaque instability. Treatment with PKT100 demonstrated notable benefits on atherosclerotic plaque stabilisation, including a reduction in intraplaque hemorrhage and neovascularisation in unstable atherosclerotic plaques. This was associated with a reduction in lipid-rich intraplaque content, irrespective of an effect on plasma lipid concentration. This is suggestive of a notable shift towards a stable plaque phenotype linked with PKT100 treatment. These findings highlight the potential of P2X7R antagonism as a targeted pharmacotherapeutic strategy in stabilising atherosclerotic plaques. Immunomodulation through P2X7R may hold significant promise for prevention against MI and stroke, offering an effective strategy in the management and treatment of coronary artery disease.

Introduction

Atherosclerosis, with ensuring unstable plaque, is a significant cause of acute myocardial infarction (MI) and stroke and the leading cause of morbidity and mortality worldwide. (1) Despite substantial improvements in primary and secondary heart attack prevention through targeting standard modifiable risk factors (hypercholesterolemia, hypertension, diabetes mellitus and smoking), there remains a significant component of disease susceptibility that continues to elude effective intervention. This is reflected by the 14-27% of first-time MI patients who exhibit no significant modifiable risk factors. (2–5) It is now well established that inflammation plays an integral role in atherosclerotic plaque progression and rupture, with recent human trials demonstrating the clinical benefits of immune modulation in preventing cardiovascular mortality. (6–8) Pivotal evidence from

the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) showed that cardiovascular mortality was reduced after neutralisation of a single inflammatory cytokine, interleukin-1 beta (IL-1 β), with a therapeutic benefit on total inflammation, indicated by plasma concentration of C-reactive protein (CRP). (6,9,10) This was shown to have downstream benefits on interleukin-6 (IL-6), a pro-inflammatory cytokine previously implicated in atherothrombosis and an independent predictor of stroke. (9,11–13) Despite this, the expected magnitude of benefit was not reached, and some patients showed no improvements, likely due to a residual inflammatory risk related to IL-18 and IL-6. (6) Thus, further efforts are required to find alternative therapeutic targets in the inflammatory cascade to achieve adequate suppression for the treatment of coronary artery disease (CAD).

The P2X purinoceptor receptor 7 (P2X7R) has been emphasised as a therapeutic target, given its crucial contribution to maladaptive inflammation. (14–18) Under classical assumptions, its activation is promoted by the accumulation of adenosine-5'-triphosphate (ATP) released in response to cellular stress. However, other inflammatory molecules are now recognised to activate the receptor. (17,18) Particularly consequential is its regulation over IL-1 β maturation and release, along with other members of the interleukin-1 family, through downstream P2X7R-dependent assembly of the NOD-like receptor protein 3 (NLRP3) inflammasome and the subsequent activation of caspase-1. (19–22) Palmitoylation of the cysteine residues in the cytoplasmic domain differentiates the P2X7R pharmacologically from other P2X members. Specifically, the palmitate groups impede P2X7R desensitisation to endogenous ligands and prevent inflammatory resolution in chronic disease states like atherosclerosis. (23) The expression of the P2X7R was identified in human carotid atherosclerotic plaques close to intraluminal thrombi and inflammatory infiltrates. (24,25) Soon after MI, P2X7R expression was upregulated at the transcriptional level in peripheral blood mononuclear cells and correlated with the degree of coronary stenosis. (26) In a recent paper, peripheral blood mononuclear cell derived monocytes from patients with CAD demonstrated heightened IL-1 β release after stimulation with 2'(3')-O-(4-Benzoylbenzoyl) adenosine 5'-triphosphate (BzATP). (15) Elevated IL-1 β could be diminished to near baseline concentrations after treatment with the P2X7R antagonist, PKT100.

Given this preliminary evidence, this study evaluates the therapeutic benefit of PKT100 in a preclinical model of atherosclerotic plaque instability. To address this question, a murine model of plaque instability was utilised to emulate the features of atherosclerosis plaque destabilisation through surgical ligation of the right carotid artery, termed 'tandem stenosis' (TS). (27–29) Apolipoprotein E deficient (ApoE^{-/-}) mice receiving a high-fat diet ad libitum for 7 weeks underwent a single procedure where tandem stenosis was induced, and an osmotic minipump was subcutaneously implanted to deliver either PKT100 or vehicle for 6-weeks. PKT100-treated mice demonstrated a substantial reduction in plaques associated with plaque destabilisation in unstable plaque segments. This included, meaningful reductions in intraplaque lipid contents, irrespective of a decrease in plasma cholesterol. Taken together, these results suggest that P2X7R antagonism may be a promising

therapeutic avenue in the treatment of atherosclerotic plaque rupture, potentially reducing the risk for MI and stroke.

Methods

For animal experiments completed at the Alfred Research Alliance, the Animal Ethics Committee of the Alfred Research Alliance, Australia, approved all animal experiments, under ethics application number E/1904/2019/B, P8275. For the experiments completed at the University of Sydney, the animal ethics at the University of Sydney, Australia approved all animal experiments under ethics application number 2022/2123. Experimental procedures were conducted under the National Health and Medical Research Council of Australia research guidelines and the Australian Code for the Care and Use of Animals for Scientific purposes.

Preparation of P2X7R antagonist (PKT100)

PKT100 is a selective and potent P2X7R antagonist belonging to a class of adamantanyl-substituted benzamide structures synthesised by Wilkinson et al. (30,31) Its structure, synthesis, and pharmacokinetic properties are reported elsewhere. (30,31) Based on the prior assessment, PKT100 was formulated at 0.2 mg/kg in 50% DMSO, 35% saline, and 15% ethanol. Mice allocated to vehicle treatment received a micro-osmotic minipump with the exact proportions of DMSO, saline, and ethanol listed above without PKT100. Micro-osmotic minipumps were filled as described in accordance with the manufacturer's instructions. A total volume of 100 µL to be delivered over 28 days at 3.84 µL per day. The day prior to implantation, micro-osmotic minipumps were filled with their respective treatment and primed overnight in sterile 0.9% saline at 37°C. Any osmotic minipumps that floated in saline were refilled to remove air bubbles to ensure adequate drug perfusion. Micro-osmotic minipumps were replaced every 4-weeks to ensure continuous delivery of PKT100.

Mouse experiments

Animals

In cohort one, the efficacy of PKT100 to improve features of plaque stabilisation was evaluated in the TS model. Male mice between 5-8 weeks of age on a ApoE^{-/-} C57BL/6J background (*B6.129P2-ApoEtm1Unc/J*) were acquired from the Animal Resource Centre (Australia). Mice experiments were conducted at the bioresource facilities at the Baker Heart and Diabetes Institute (Australia) and the Kearns Facility at the University of Sydney (Australia). Tandem stenosis was induced, as previously described. (1) Mice that underwent TS received a high-fat diet composed of 21% fat and 0.15% cholesterol (Specialty Feeds, SF00-219, Australia) and distilled water (pH 7.2) ad libitum for a total of 13 weeks from 6-9 weeks of age. Six weeks after high-fat diet commencement (between 12-15 weeks of age), mice were anaesthetised with a combination of 100 mg/kg ketamine (Troy Laboratories, Australia) and 10 mg/kg xylazine (Troy Laboratories, Australia) through intraperitoneal injection. Mice sustained TS in which the right carotid artery is inflicted with two stenoses placed 1 mm from the bifurcation and 3 mm from the initial ligation to induce an unstable atherosclerotic phenotype. The stenosis was made with non-absorbable 8-0 virgin silk sutures fitted with a 32-gauge swaged needle (Johnson & Johnson,

USA) After the procedure, mice were placed in a prone position and a lateral cut was made in the interscapular region and undermined to create a subcutaneous pocket. A micro-osmotic minipump was implanted subcutaneously, either containing 0.2 mg/kg PKT100 or vehicle treatment for 7 weeks (Alzet model 2004, Germany). The study was undertaken blinded, with the treatment groups unknown throughout the study. All animals reached treatment endpoints. In a second cohort, the efficacy of PKT100 to attenuate atherosclerotic plaques was evaluated. In short, 6-9-week-old mice ApoE^{-/-} C57BL/6J background (*B6.129P2-ApoEtm1Unc/J*) received from the Australian Resource Centre (Australia) received a high-fat diet from 8-weeks of age (composed of 21% fat and 0.15% cholesterol, Australia). At the same timepoint, an osmotic minipump was subcutaneously embedded as described above to deliver 0.2 mg/kg or vehicle for 13 weeks.

Blood collection

At the end of the study, mice were terminated with 300 µg/g ketamine (Troy Laboratories) and 30 µg/g xylazine (Troy Laboratories) under non-fasted conditions. Whole blood was collected through cardiac puncture with a 1 mL syringe and 100 µL of Clexane (LMWH) 400 IU. The vasculature was perfused with phosphate buffer saline. Blood maintained on wet ice (4°C) was centrifuged at 2000 *xg* for 15 minutes at 4°C with a break on. Plasma was collected and stored at -80°C until subsequent analysis.

Sysmex haematology analysis

Blood was diluted 1:7 in Cellpack (Biolab Diagnostics, Italy) for a final volume of 100 µL. Samples were homogenised and assayed on the Sysmex (XS-1000i/XS-800i, Wakinohama-Kaigandori, Japan) in Capillary mode to capture, white blood cell counts, erythrocytes, hematocrit, platelets, neutrophils, lymphocytes, and monocytes. The neutrophils, lymphocytes, and monocytes were reported as the proportion of the total white blood cell count.

Plasma biomarkers

Systemic markers of inflammation were measured from plasma collected. A list of these markers is reported in the supplementary material. The markers were assessed by a ProcartaPlex antibody and magnetic-based multiplex immunoassay (Luminex xMAP) per the manufacturer's instructions (Thermo Fisher Scientific, USA). Briefly, the capture antibodies provided were conjugated to metal beads, each with a distinct colour shade for detection. Plasma samples were incubated with the beads in a universal dilution buffer for 2 hours on a shaker set to 600 *xg* at room temperature. The beads with captured analytes were washed once with wash buffer before the plate was quickly inverted and blotted on an absorbent towel. The beads are then incubated with streptavidin-R-phycoerythrin. The beads were incubated for 30 minutes on a shaker set to 600 *xg* at room temperature. The plate was then washed three times with wash buffer as previously described and in the final step was blotted on an absorbent towel. Following a final wash step, the beads are resuspended in a universal resuspension buffer for analysis. Before reading, beads were resuspended through a 5-minute 600 *xg* shake at

room temperature. Wells were read on a Luminex MAGPIX Multiplate reader (Thermo Fisher Scientific) at 532 and 635 nm. Data was exported as a CSV file and analysed in Excel as outlined in the methods.

Tissue collection

After vasculature perfusion, the right common carotid artery was isolated and cut into segments I and II. Segment I was prioritised for assessment as it is characterised as the unstable segment due to its proximity to the first ligation. (27) The left common carotid artery, thoracic and abdominal aorta, and aortic root were also isolated and embedded in Optimal Cutting Temperature (Tissue-Tek, USA), gradually frozen on dry ice, and stored at -80°C before assessment. From a portion of the mice, the aorta was removed, fixed in 10% neutral buffered formaldehyde for 4 hours, and later the visceral fat was removed. In preparation for en face staining, the aorta was cut down the middle and pinned to silicon for assessment. From these mice, the brains were also removed after perfusion and immediately fixed in 10% neutral buffer formalin for 6-hours on ice. After fixation, the brains were moved into a 30% sucrose stock for cryopreservation and placed in the fridge until assessment. Other tissues were collected for basic measurements and downstream molecular function. The weight of organs was normalised to the tibia³ length for each mouse. This measure shows a strong linear correlation with healthy mice weight. In this model, where body weight is affected, organ weight relative to body weight becomes a poor predictor of dysfunction. Normalising to the tibia length provides a more accurate measure of organ size between treatment groups.

Histology

Segment I of the RCA was sectioned 300 µm, 600 µm, and 900 µm from the first stenosis on an NX70 cryostat (Thermo Fisher Scientific) set a thickness of 7 µm. Picrosirius red staining was performed to visualise collagen deposition as a marker of plaque stabilisation using the previously described methods. (27) In short, frozen sections were brought to room temperature for a minimum of 1 hour before being fixed in 10% neutral buffered formalin for 10 minutes at room temperature. Samples were incubated for 1 hour in PSR solution (Sigma-Aldrich, USA) which was composed of 0.6% w/v direct red 80 reconstituted in 1.3% Picric acid saturated in water. Samples were then rinsed in water and differentiated in 0.1 M hydrochloride acid. Slides were mounted with DPX mounting media (Sigma-Aldrich). Hematoxylin and eosin (Picrosirius red) staining was used as a quantification of the plaque size, and morphological assessment of plaque burden and necrotic core. The necrotic core was defined as previously described. (32,33) Briefly, the necrotic core was identified by plaque areas with a complete loss of nuclei measured by hematoxylin and eosin and a loss of extracellular matrix collagen from picrosirius red segments. Intraplaque lipid content was measured from Oil red O staining (Sigma-Aldrich).

Immunohistochemistry

To quantify atherosclerotic intraplaque hemorrhages, neovascularisation, macrophage infiltration and smooth muscle cell disposition, immunohistochemistry was performed as previously described. (27) The antibodies used

were anti-CD31 (clone MEC13.3; 1:100 dilution; R&D Systems; anti-SMA (clone 1A4; 1:100 dilution, Sigma-Aldrich); rat anti-mouse CD68⁺ (MCA1957GA; 1:100); and TER119 (13-5921-81; 1:400, eBioscience). Briefly, tissue sections were fixed in ice-cold acetone and left to dry for 20 minutes at room temperature. Samples were subsequently blocked with a blocking buffer containing 1% BSA in PBS for 30 minutes at room temperature. Primary antibodies were reconstituted in the same blocking buffer and incubated on samples for one hour at room temperature to achieve protein detection. Detection was achieved using the Vectastain ABC (Thermo Fisher Scientific) kit and 3,3'-diaminobenzidine (Thermo Fisher Scientific) substrate for CD31, CD68⁺ and anti-SMA. For the detection of TER119, sections were incubated with a biotinylated anti-mouse TER119 antibody (1:400) in phosphate buffer solution overnight at 4°C. Per manufacturer instructions, sections were warmed to room temperature, washed with provided washing buffer, and incubated with avidin-biotin complex and visualised with 3, 3'-diaminobenzidine substrate (Sigma-Aldrich). The specificity of rat antibodies was achieved with rat IgG2B control to validate the stain. Other isotype control antibodies were used to validate each immunostaining according to the primary antibody. All images were captured on an Olympus BX41 (20x objective) using an Olympus DP22 camera.

Images and analysis

For histological and en face stain, images were captured on an Olympus BX41 with a 20 times objective fitted with an Olympus DP22 camera. All histological photos were analysed in Fiji, open source image processing package based on ImageJ2. (34) In particular, Oil red O and plaque necrotic areas were assessed at red or white threshold levels. Plaque collagen determined by PSR quantification was based on red, green, and yellow polarised light and adjusted based on consistent thresholds. The percentage of intimal area covered by red stain indicative of atherosclerotic lesions was calculated by Gimp v 2.10.29 software.

Plasma lipid analysis

A complete blood lipid profile was captured with a Cobas Integra® 400 plus autoanalyser using the following standard commercial enzymatic assay kits (Roche Diagnostics, Germany): cholesterol Gen.2, HDL-cholesterol plus 2nd generation, LDL-cholesterol plus 2nd generation, and triglycerides. Colorimetric changes were measured at 512 nm for total cholesterol and triglycerides or at 583 nm for high-density lipoprotein (HDL) and low-density lipoprotein (LDL).

En face for intimal plaque area

Total atherosclerotic burden was determined in the aorta using the en-face method. As referenced above, in a proportion of mice from the study, the whole aorta was immediately removed and cleaned of the visceral fat before being fixed in 10% neutral buffered formalin overnight. Aortas were stained with 0.5% Oil red O in isopropanol (Sigma-Aldrich) for 20 minutes at room temperature, then washed under gently running tap water

for 1 hour. Before being pinned out, any remaining branches were removed from the arch and then slit down the middle. Aortas were pinned out flat on dental wax with entomology pins.

Statistical analysis

All data is presented as mean $\pm$ SEM. Unless otherwise indicated, all data was analysed by a parametric t-test (Prism v.9.1.0). A P value of <0.05 was considered statistically significant. All data was analysed blinded by two independent researchers. In this case, the drug formulation was prepared in advanced at the University of Sydney and a code was provided. Two independent analyses of the histological images were conducted and were later confirmed.

Results

PKT100 was safe and had no adverse consequences on neutrophils

In chapter three, peripheral blood mononuclear cell derived monocytes isolated from participants with CAD exhibited heightened IL-1 β production upon stimulation with the potent synthetic agonist BzATP, in contrast to monocytes from patients without CAD. (15) This observed release was particularly evident in those with calcified CAD. (15) Given the substantial contribution of inflammation in atherosclerotic plaque rupture, the potential efficacy of PKT100 was evaluated in a mouse model of atherosclerotic plaque destabilisation. 7-weeks after HFD commencement, mice underwent simultaneous right carotid artery ligation – referred to as TS. In the same procedure, mice also received either subcutaneous implantation of an osmotic minipump containing PKT100 or vehicle for 6 weeks (**Figure 1A**). No adverse events were reported throughout the study. Body weight was increased by 9.6% after 7 weeks of HFD treatment (27.38 ± 0.33 vs 28.50 ± 0.38 , $p=0.013$). Systemic delivery of PKT100 for 6 weeks had no effect on body weight between groups (28.0 ± 0.40 vs 28.78 ± 0.42 , $p=0.380$) (**Figure 1B**). Considering that pharmacotherapies with notable impact on immune function tend to obliterate circulating immune populations (2,19), the effect of PKT100 on immune cell counts was evaluated. Treatment with PKT100 significantly increased neutrophils counts compared to vehicle-treated mice (142.1 ± 19.54 vs 233.3 ± 22.45 , $p=0.0046$) (**Figure 1C**). There were no observable differences in other leukocyte populations (**Figure 1D-E**). While the weight of the heart, kidney, and spleen did not differ between PKT100 and vehicle-treated mice, liver weights were notably higher in the PKT100 group when adjusted based on the tibia³ length (**Figure H-M**).

PKT100 treatment ameliorates intraplaque hemorrhage and neovascular formation

The presence of intraplaque hemorrhages in human carotid atherosclerotic plaque is a feature of plaque instability. (35–37) To determine whether PKT100 can exert plaque stabilisation effects in vivo, TER119, an erythrocyte marker and feature of intraplaque hemorrhage was evaluated. PKT100 reduced intraplaque hemorrhage by 0.46-fold compared to mice treated with vehicle alone ($7.88\% \pm 1.13$ vs $3.66\% \pm 0.64$, $p=0.0037$) (**Figure 2A**). Next, CD31, a surface endothelial marker was used to measure the formation of neovessels that have demonstrated a deleterious role in atherosclerotic plaque instability. Treatment with PKT100 was accompanied by a 0.44-fold reduction in neovascularisation compared to the vehicle-treated group

($1.673\% \pm 0.28$ vs $0.7319\% \pm 0.13$, $p=0.0088$) (**Figure 2B**). These results indicate that inhibiting P2X7R might contribute to stabilisation of rupture-prone atherosclerotic lesions. Previous clinical trials with anti-inflammatory agents such as Canakinumab demonstrated notable reductions in systemic inflammatory inhibition through single cytokine neutralisation. (6,9) Under these circumstances, systemic inflammatory markers were measured from mouse blood at the end of the study. CRP was evaluated due to its utility as a surrogate marker in other anti-inflammatory trials. There were no significant differences in plasma CRP levels between mice treated with PKT100 or vehicle (**Figure 2E**). Other more upstream markers such as IL-6, IL-18 and IL-1 β were also measured given their downstream modulation by the P2X7R. There were notable differences in systemic markers (**Figure 2F-H**). Despite the lack of reduction in systemic inflammatory markers, mice treated with PKT100 demonstrated a significant improvement in plaque stabilisation.

PKT100 reduces lipid-rich extracellular matrix irrespective of a change in plasma lipid measures

The presence of lipid-rich necrotic cores in atherosclerotic plaques has been shown to be pivotal in predicting rupture risk. (32,38,39) To further examine the impact of PKT100 on extracellular matrix composition in atherosclerotic plaques, plaque lipid composition was evaluated with Oil Red O, a diazo dye that stains triglycerides and lipids. Mice treated with PKT100 demonstrated a 33.2% reduction in plaque lipid composition compared to the vehicle-treated group ($39.57\% \pm 2.10$ vs $26.43\% \pm 2.99$, $p=0.004$) (**Figure 3A**). This finding is consistent with a shift towards a more stable phenotype in response to P2X7R antagonism. In alignment with these observations, PKT100 was further evaluated for its ability to exert a beneficial effect on lipid accumulation in stable atherosclerotic plaque segments. Mice treated with PKT100 demonstrated a 17.8% reduction in lipid-rich necrotic cores in aortic sinus plaques, regions that are away from the turbulent conditions driven by the tandem stenosis model ($47.0\% \pm 3.06$ vs $38.63\% \pm 2.45$, $p=0.051$) (**Figure 3B**). Although these results did not meet the predefined criteria for statistical significance, they suggest that the P2X7R might be critical in lipid transport exacerbated by 'unstable' conditions. To understand whether PKT100 exerted its plaque stabilisation effects through modifying plasma lipids, total cholesterol, triglycerides, HDL, and LDL concentrations were measured from plasma. There were no detectable differences in plasma lipid levels in PKT100-treated mice compared to the vehicle group (**Figure 3E-G**). These results predict a potential role for P2X7R-mediated inflammation in lipid transport exacerbated in unstable atherosclerotic conditions.

Plaque volume and progression in the aorta remote from the tandem stenosis

Previous studies have demonstrated that atherosclerotic plaque volume was reduced in mice deficient in the P2X7R (40,41). Plaque volume was not measured from stenotic regions, as the manual constriction of the vessel factitiously reduced its internal diameter. For this reason, plaque progression in the aorta was examined to determine whether there were potential changes in atherosclerotic plaque size which drove plaque stabilisation in unstable segments. No significant difference was observed in the total aortic plaque burden ($4.71\% \pm 0.72$ vs $6.84\% \pm 0.68$, $p=0.160$) or in the abdominal aorta ($13.47\% \pm 2.0$ vs $16.42\% \pm 2.02$, $p=0.326$) between groups (**Figure 4A-C**). Given the inconsistent with previous literature PKT100, the potential role of PKT100 in plaque

attenuation was further evaluated in a mouse atherosclerosis model without tandem stenosis. Male ApoE^{-/-} mice were fed a high-fat diet for 13-weeks to recapitulate the timeline in the tandem stenosis study. Concomitantly, mice received either PKT100 or vehicle from the onset of the high-fat diet (**Figure 4D**). Given the relatively low plaque burden in the right carotid in this model, atherosclerotic plaque size in the brachiocephalic trunk was assessed. There were no notable differences in lesion area between PKT100-treated mice compared to the vehicle group ($54.79\% \pm 6.71$ vs $56.40\% \pm 3.03$, $p=0.830$) (**Figure 4E-F**). Although these results did not replicate the previous findings in mice P2X7R deficient mice, they indicate that plaque stabilisation occurs independently of a reduction in plaque development.

In the clinical setting, the detection of atherosclerotic plaque depends on the identification of calcified lesions with computerised tomography. (42,43) By the time these lesions are detected, they are often considered advanced. (42) Intraplaque collagen has a bilateral role in atherosclerosis, where its expression is associated with plaque stabilisation through mechanical resilience but may cause vascular occlusion. (44) To elucidate whether PKT100 can influence collagen deposition, intraplaque collagen was measured in unstable atherosclerotic plaque segments. Quantification of total plaque burden in mouse atherosclerotic lesions did not discriminate discernible differences in collagen deposition between the PKT100 and vehicle-treated groups ($8.58 \pm 1.5\%$ vs $6.6 \pm 1.95\%$, $p=0.58$) (**data not shown**). However, a clear trend becomes apparent when evaluated as a proportion of mature collagen fibres compared to immature fibrin repositioned based on colour spectrums. Under polarised light, picrosirius red enhances the natural birefringence of collagen, which appears as green, yellow and red fibres on a dark background. Mice treated with PKT100 demonstrate a larger proportion of mature collagen, characterised by a thick and well-established orange-to-red matrix, compared to the thin and immature fibres denoted in the yellow-to-green (2.2 ± 0.08 vs 1.9 ± 0.04 , $P=0.016$) (**Figure 4G-H**). These results suggest an effect on plaque stabilisation through increased mechanical stiffness by mature collagen networks in the extracellular matrix.

PKT100 is not associated with a reduction in macrophage infiltration

Macrophages abundantly express P2X7R and have emerged as critical orchestrators in necrotic core formation. (45–48) As plaque progresses to a more unstable phenotype, pro-inflammatory macrophages are recruited to lesions to remove apoptotic cells. (47) Given the notable trends towards a more stable phenotype associated with PKT100 treatment, without effects on systemic inflammation, it was hypothesised whether this mechanism might be mediated by macrophage infiltration at the atherosclerotic lesion. Quantification of the unstable segment showed no observation differences in CD68⁺ macrophage expression in between groups ($27.1 \pm 2.15\%$ vs $24.9 \pm 2.04\%$, $p=0.45$) (**Figure 5A**). However, immunomodulation in atherosclerosis can be complicated by the diversification of hematopoietic and non-hematopoietic populations in response to cellular crosstalk in the plaque microenvironment. Recent evidence has shown that SMCs can differentiate into macrophage-like cells under certain conditions and contribute to plaque progression. (49,50) Under these circumstances, it was more broadly explored whether PKT100 treatment was associated with changes in SMC expression, denoted by alpha-

smooth muscle actin (α -SMA) stain in unstable segments. There was no difference in the detection of intraplaque α -SMA between PKT100 and vehicle-treated groups ($5.55 \pm 1.07\%$ vs $4.90 \pm 0.75\%$, $p=0.64$) (**Figure 5B**). Although, previous studies have also pointed to the relevance of the macrophage and SMC ratio to delineate stability in atherosclerotic lesions. Atherosclerotic plaques with a higher macrophage to SMC ratio was associated with greater plaque instability. The ratio between macrophages and SMCs was measured to evaluate whether unstable segments from PKT100 demonstrate notable features of stable atherosclerotic plaques compared to vehicle-treated mice. There was no significant difference was detected in the macrophage to SMC ratio between PKT100 and vehicle groups (7.85 ± 1.17 vs 6.21 ± 0.83 , $p=0.28$) (**Figure 5C**).

Discussion

Here, this study provides novel insights into the benefits of targeting the P2X7R in a murine model of plaque instability. Building upon the previous findings in chapter three which showed heightened P2X7R-dependent IL-1 β release could be diminished with PKT100 in monocytes from patients with CAD, this study sought to elucidate whether targeting P2X7R could be an effective management strategy in plaque stabilisation. These findings showed that treatment with PKT100 may have a therapeutic benefit in plaque stabilisation. Plaques from unstable segments showed that PKT100 attenuated intraplaque hemorrhage and neovascularisation, despite no reduction in plaque size. While this mechanism was not further elucidated, it is hypothesised that the reduction in neovascularisation may have protected against intraplaque hemorrhage in the lesions. PKT100 treated mice also showed a substantial decrease in lipid-rich necrotic cores, irrespective of a change in plasma lipid concentrations. These results recapitulate clinical data which shows improvements in mortality without lipid modification. Evaluation of intraplaque collagen content also indicated a potential increase in biomechanical strength denoted by a higher proportion of mature intraplaque collagen, despite no difference in total collagen deposition. These results suggest a remarkable shift towards a stable plaque phenotype in unstable atherosclerotic segments from PKT100-treated mice.

The association between inflammation and risk of CAD, as evidenced by landmark clinical trials, has opened new avenues in drug discovery for the management of CAD. The initial interest in these biological pathways was sought after evidence from the JUPITER trial showed that statins indirectly reduced CRP by lowering plasma LDL in a population with low to moderate LDL levels. (51) Compared to previous trials, selection based on CRP concentration rather than LDL led to a greater relative benefit in primary endpoints. (51) While an increased clearance of cholesterol likely drove the substantial reductions in plasma CRP rather than a direct effect of statins on inflammatory pathways, a clear relationship was established between lipids and inflammatory signalling. The subsequent CANTOS trial demonstrated that the inflammatory response can be targeted independently of lipid modifications to improve primary outcomes. (6,9,52) This confirms that while lipids act on inflammatory pathways to promote atherogenesis, inflammation cannot facilitate or depose lipid risk. (53–55) Instead, immunomodulation of cytokines may have downstream effects on the cellular crosstalk between innate and adaptive immune systems and the plaque microenvironment.

It was shown that PKT100 reduced lipid-rich necrotic cores in unstable atherosclerotic plaque segments. This was achieved without modification to plasma cholesterol, triglycerides, or LDL: HDL ratio parameters. This is consistent with earlier findings that showed that LDL^{-/-}, P2X7R^{-/-} mice demonstrate a reduction in lipid accumulation in atherosclerotic lesions. (40) These destabilised regions form in response to defective efferocytosis, where macrophages are overwhelmed by the constant demands of lipid clearance. (45,53,56) Their final fate in atherosclerotic lesions is apoptosis, where their ruptured bodies form the foundations of a weakened necrotic core. Lipid-rich necrotic cores within atherosclerotic plaques are independently associated with plaque destabilisation, as indicated by fibrous cap status. (32,39,57)) It was suspected that immunomodulation may regulate crosstalk between cellular populations and determine the fate of macrophages in plaque. At this time, this hypothesis has yet to be evaluated. Despite their efficacy in reducing plasma cholesterol, statins do not demonstrate discernible benefits on intraplaque lipid accumulation (27) unless received at a concentration 2-4-fold higher than standard dosages. (43,58)

There was also a substantial benefit on intraplaque neovascularisation and intraplaque hemorrhage in mice treated with PKT100. Other murine atherosclerosis models fail to recapitulate features of neovascularisation and intraplaque hemorrhage in atherosclerotic plaques, given their resistance to plaque destabilisation (27) Chen et al. previously showed that features of intraplaque hemorrhage and neovascularisation occurred as a result of alterations in shear and tensile stress. (27) Neovascularisation is thought to be an extension from the adventitia into the media, driven by vascular endothelial growth factor to meet the oxygen demands inside atherosclerotic lesions. (35,59) However, these vessels are often immature, denoted by the absence of α -SMA expression, despite CD31 expression. (36) Similarly to cancerous lesions, vascularisation in atherosclerotic plaque facilitates the infiltration of inflammatory mediators and the deposition of erythrocytes that form intraplaque hemorrhages. (37,60) The breakdown of erythrocyte membranes from intraplaque hemorrhages are proposed to be an independent risk factor for MI, and are associated with a 'soft' plaque phenotype that are proposed to be more unstable than established calcified plaque.

Considering the substantial reductions in plaque burden observed in LDL^{-/-}, P2X7R^{-/-} mice—a result that was replicated in an APOE^{-/-}, siRNA P2X72^{-/-} by an independent group (41), there were no discernible differences in plaque burden between in this study. The discrepancies between these findings and previously published literature may indicate a potential limitation of PKT100 efficacy in vivo. This may be explained by data in chapter three, which showed an 85% reduction in IL-1 β release from monocytes treated concurrently with 100 μ M BzATP and 1 μ M PKT100. Compared to the ex vivo study, concentrations were selected to demonstrate the absolute benefits of IL-1 β reduction based on the half-maximal inhibitory value on THP-1 cells. The concentrations chosen for in vivo assessment were considered to be low.

Moreover, variations in the therapeutic effect of P2X7R in vivo may stem from the preference of PKT100 to act as a non-competitive allosteric modulation. This preference for allosteric modulation may affect its ability to suppress inflammation in vivo. This is supported by evidence from the rat P2X purinoceptor 7 crystal structure, which shows that the region involved in antagonist-receptor interaction is distinct from the ATP-binding. (61) Bound ATP facilitated a conformational change to the P2X7R, concealing the area required for allosteric inhibition. (23,61) GSK-1482160, a known negative allosteric modulator of the P2X7R, failed to reach adequate inhibitory concentrations within safety margins for sufficient target engagement. (62) Thus, the efficacy of a P2X7R antagonist might be improved with a promiscuous compound that can occupy both the orthosteric and allosteric sites. However, further studies are required to identify whether binding occupancy can affect in vivo efficacy.

Despite considerable strides in the discovery of pharmacotherapies that mediate inflammatory pathways involved in plaque destabilisation, translation of these compounds has been impeded by unspecific targets with unfavourable safety profiles. (6,8,63) Given the unique pharmacological profile of the P2X7R, targeting the receptor offers several advantages over traditional immunomodulatory therapies. Extracellular ATP, an agonist of the P2X7R is required in micromolar to millimolar concentrations to exert 50% of the maximal response. Indeed, the high concentrations needed to elicit receptor activation distinguish it from other purinergic receptors. (64) Extracellular ATP can originate from the released contents of apoptotic cells under pathogenic conditions. This activation may be particularly relevant in atherosclerotic plaque destabilisation, where dysfunctional cells promote the continuous release of pro-inflammatory cytokines. This can be evidenced by the internal mammary artery, where intimal growth and plaque development are considered unusual. (24) While the biological basis for this unique phenotype is dependent on differences in endothelial nitric oxide regulation, the internal mammary artery also lacks expression of P2X7R and IL-1 β . (65,66) This expression is restored in unstable and vulnerable human atherosclerotic plaques. These diseased vessels exhibit heightened expression of IL-1 β that could be mitigated after incubation with A-740003, a selective P2X7R antagonist developed by Abbott Laboratories. (24) Thus, it is suggested that targeting IL-1 β through a P2X7R-dependent mechanism would confer protection against 'sterile' tissue-specific injury, preserving host-immunity defence against invading pathogens. Although a detailed mechanism for P2X7R in atherosclerotic plaque destabilisation has yet to be determined, it was demonstrated that P2X7R antagonism remains an effective therapeutic strategy for atherosclerosis.

Limitation

A current limitation of this study is the delivery methods and single treatment concentration. Commercialised compounds benefit from easy administration, with oral administration being preferred. In this instance, PKT100 was delivered through a subcutaneously implanted osmotic minipump to achieve prolonged plasma concentrations. To assess potential translatability, future experiments require testing to be conducted through oral administration with gavage or with the compound reconstituted in food. Moreover, in this study, the

influence of only a single concentration of PKT100 on plaque instability was evaluated. Future studies must assess the drug's efficacy in atherosclerosis across various concentrations. While the application of the tandem stenosis model better recapitulates the clinical features of destabilised atherosclerotic plaque compared to other atherosclerotic models, the model can only induce thrombosis in a small number of mice. We, therefore, cannot assess whether PKT100 can reduce thrombi that may elicit strokes in treated groups.

Conclusion

In this study, a murine model designed to mimic atherosclerotic plaque instability through tandem right carotid artery ligation in APOE^{-/-} mice fed an HFD was used to evaluate the efficacy of PKT100 to stabilise atherosclerotic plaques. These findings are indicative of a notable benefit in atherosclerotic plaque stabilisation. Notably, atherosclerotic lesions from PKT100-treated mice demonstrated substantial reductions in markers of (1) intraplaque hemorrhage, (2) neovascularisation, and (3) lipid-rich necrotic cores. This was achieved independently of a decrease in plasma lipids, further strengthening the association between inflammatory mitigation and treatment of ASCVD. In conclusion, this study indicates a potential therapeutic benefit of P2X7R antagonism in plaque stabilisation. Further studies are warranted to understand the translational potential in human populations.

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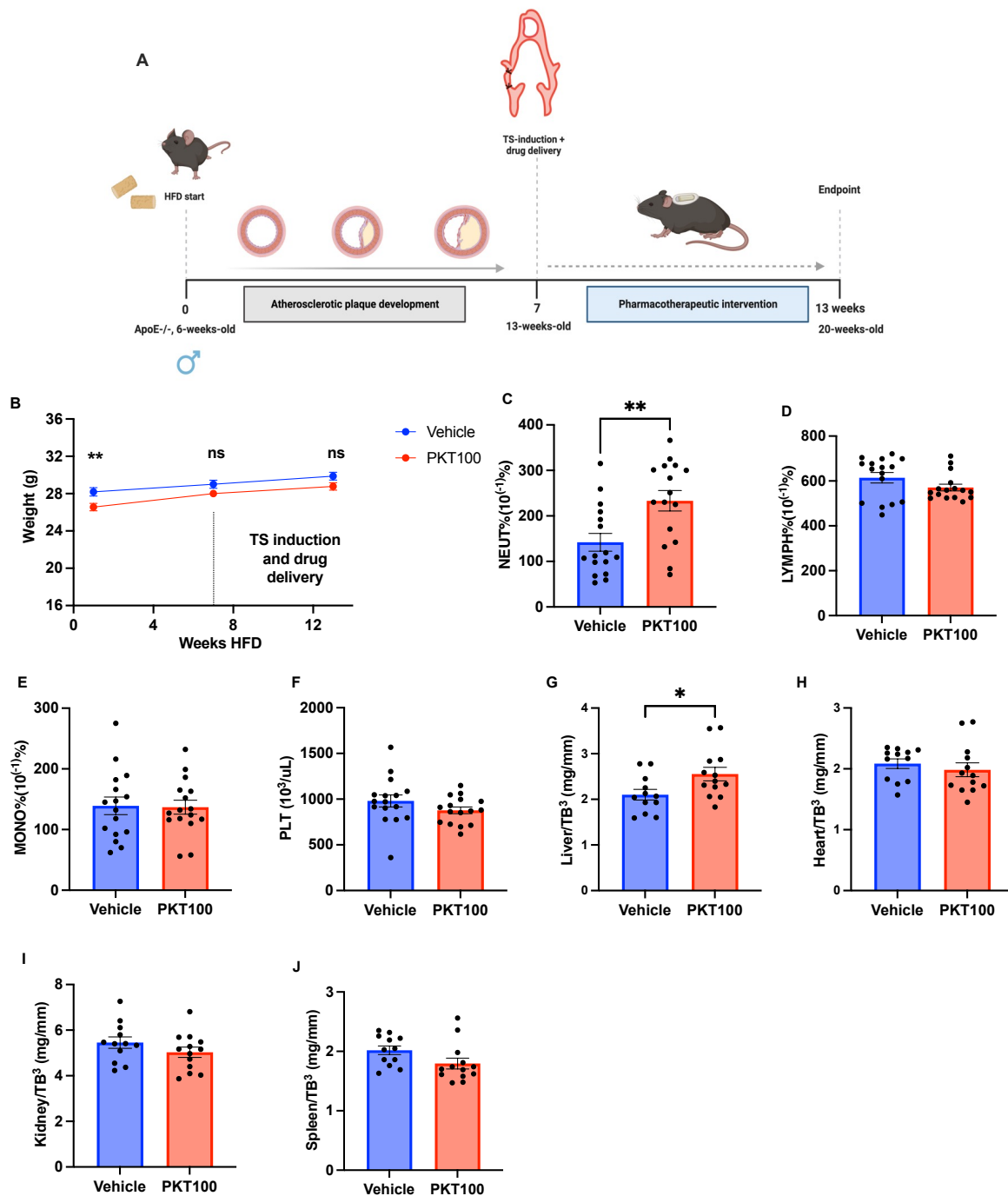


Figure 1. A murine model of atherosclerotic plaque destabilisation induced through tandem ligation of the right carotid artery. A) Schematic of the protocol overview and drug delivery procedure. **B)** Body weight measurements for the duration of the protocol (vehicle, n=16, PKT100, n=16). The proportion of **C)** neutrophils, **D)** lymphocytes, and **E)** monocytes, compared to total white blood cell count. **F)** Platelet counts from whole blood (vehicle, n=16, PKT100, n=16). Organ weights compared to tibia **G)** liver, **H)** heart, **I)** kidney, and **J)** spleen (vehicle, n=8, PKT100, n=8). Values are expressed as mean $\pm$ SEM. *P<0.05, **P<0.005. **Figure created in Biorender..**

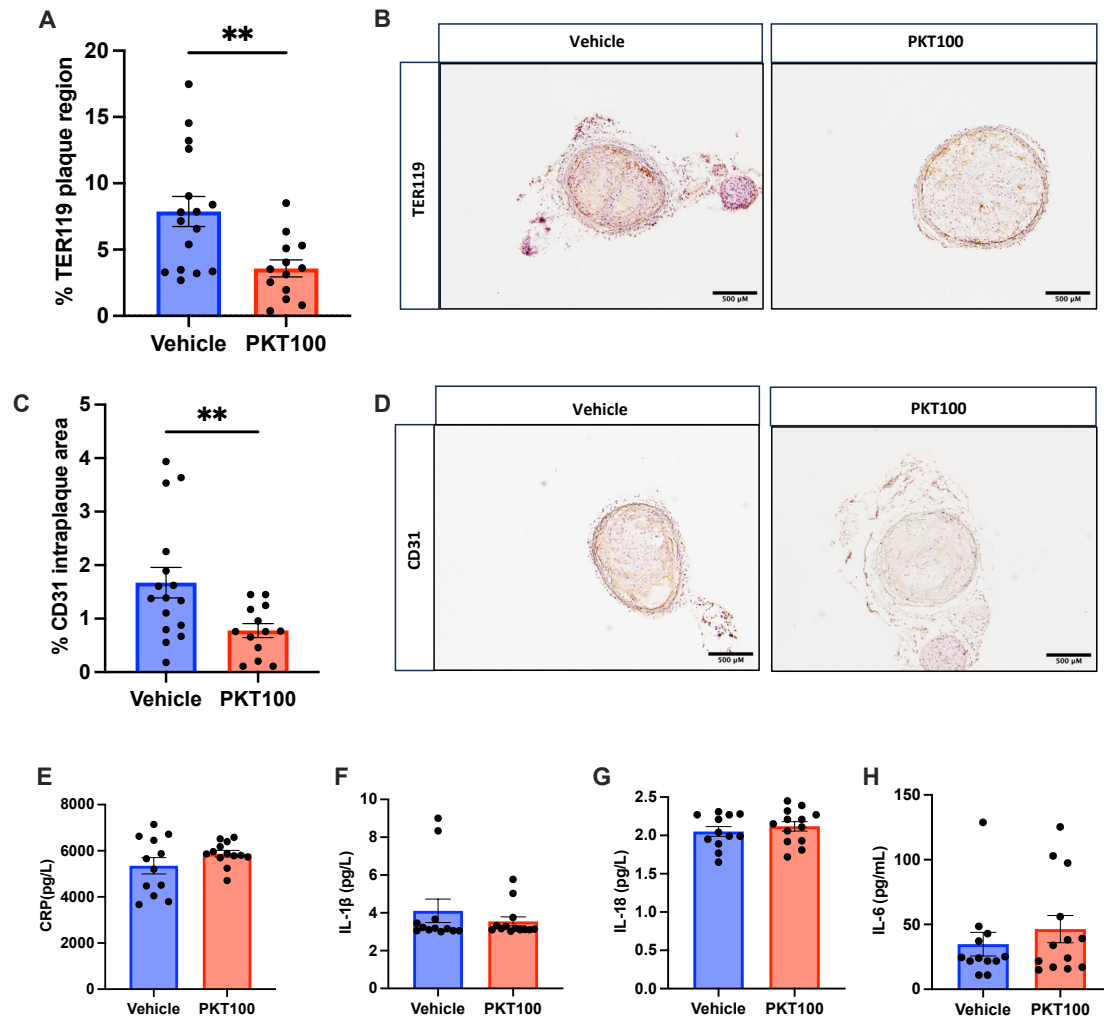


Figure 2. Intraplaque hemorrhage and neovascularisation are reduced by PKT100 treatment in unstable atherosclerotic plaque segments. **A)** Quantification of erythrocyte positive region normalised to the plaque area identified by TER119 expression (**vehicle**, $n=16$, **PKT100**, $n=13$). **B)** Representative images of TER119 expression from PKT100 and vehicle-treated mice **C)** Quantification of neovascularisation normalised to the plaque area marked by CD31 expression (**vehicle**, $n=16$, **PKT100**, $n=13$). **D)** Representative images of CD31 expression from PKT100 and vehicle-treated mice. Plasma concentrations **E)** C-reactive protein (CRP), **F)** interleukin- (IL-) 1 β , **G)** IL-18, and **H)** IL-6 (**vehicle**, $n=12$, **PKT100**, $n=13$). Values are expressed as mean $\pm$ SEM. ** $P<0.005$.

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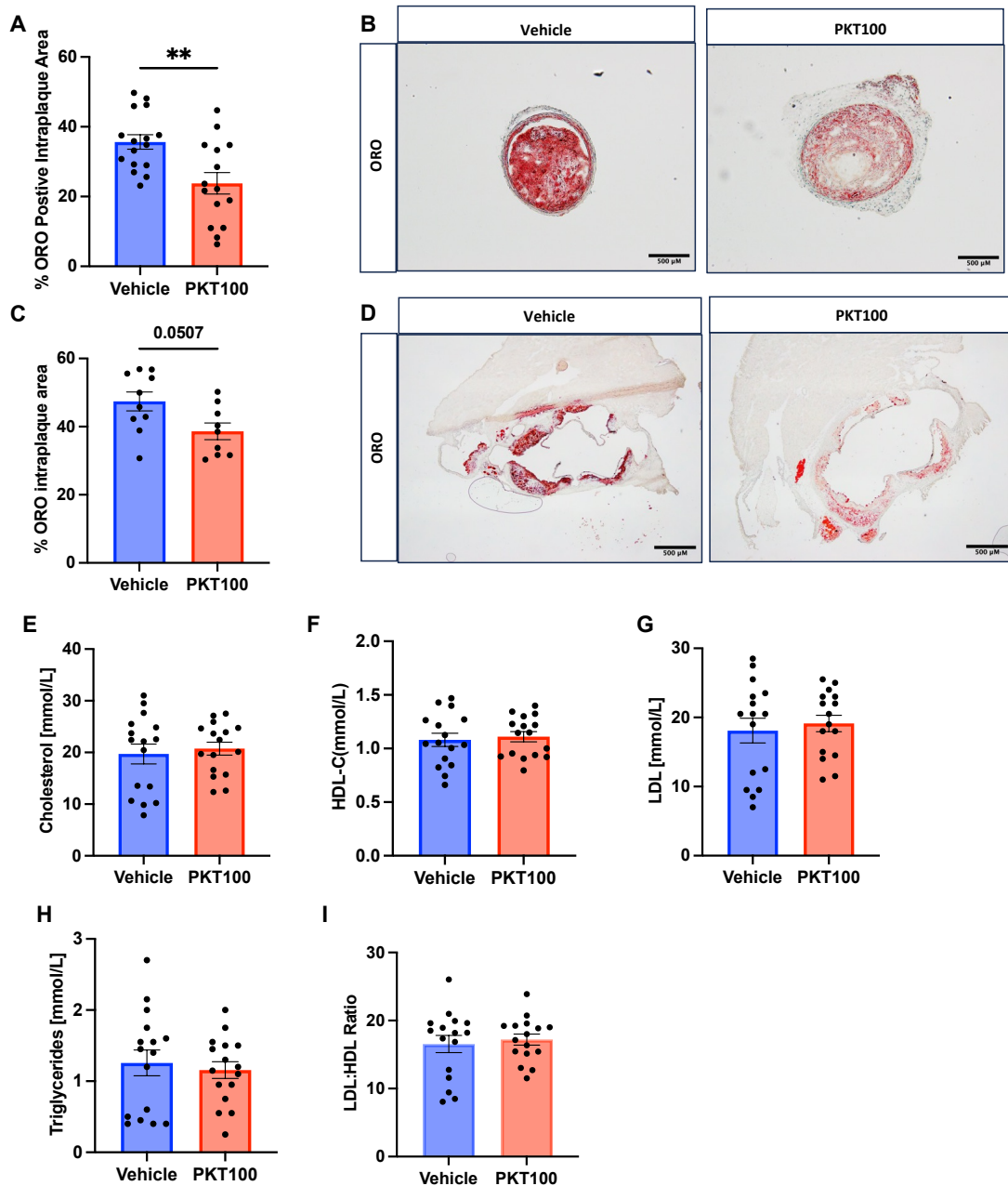


figure 3. Treatment with PKT100 reduces lipid-rich necrotic cores in unstable atherosclerotic plaque segments.

A) Quantification of Oil Red O positive region in unstable atherosclerotic plaque segments normalised to the plaque area (**vehicle**, n=16, **PKT100**, n=15). **B)** Representation of Oil Red O-positive stain from unstable atherosclerotic plaque segments from PKT100 and vehicle-treated mice. **C)** Quantification of Oil Red O-positive region in stable atherosclerotic plaque segments (aortic sinus) normalised to aortic sinus region (**vehicle**, n=10, **PKT100**, n=9). **D)** Representation of Oil Red O-positive stain in stable segments from the aortic sinus region. Plasma lipid concentrations **E)** cholesterol, **F)** HDL, **G)** LDL, **H)** triglycerides, and **I)** LDL/HDL ratio (**vehicle**, n=16, **PKT100**, n=16). Values are expressed as mean $\pm$ SEM. **P<0.005.

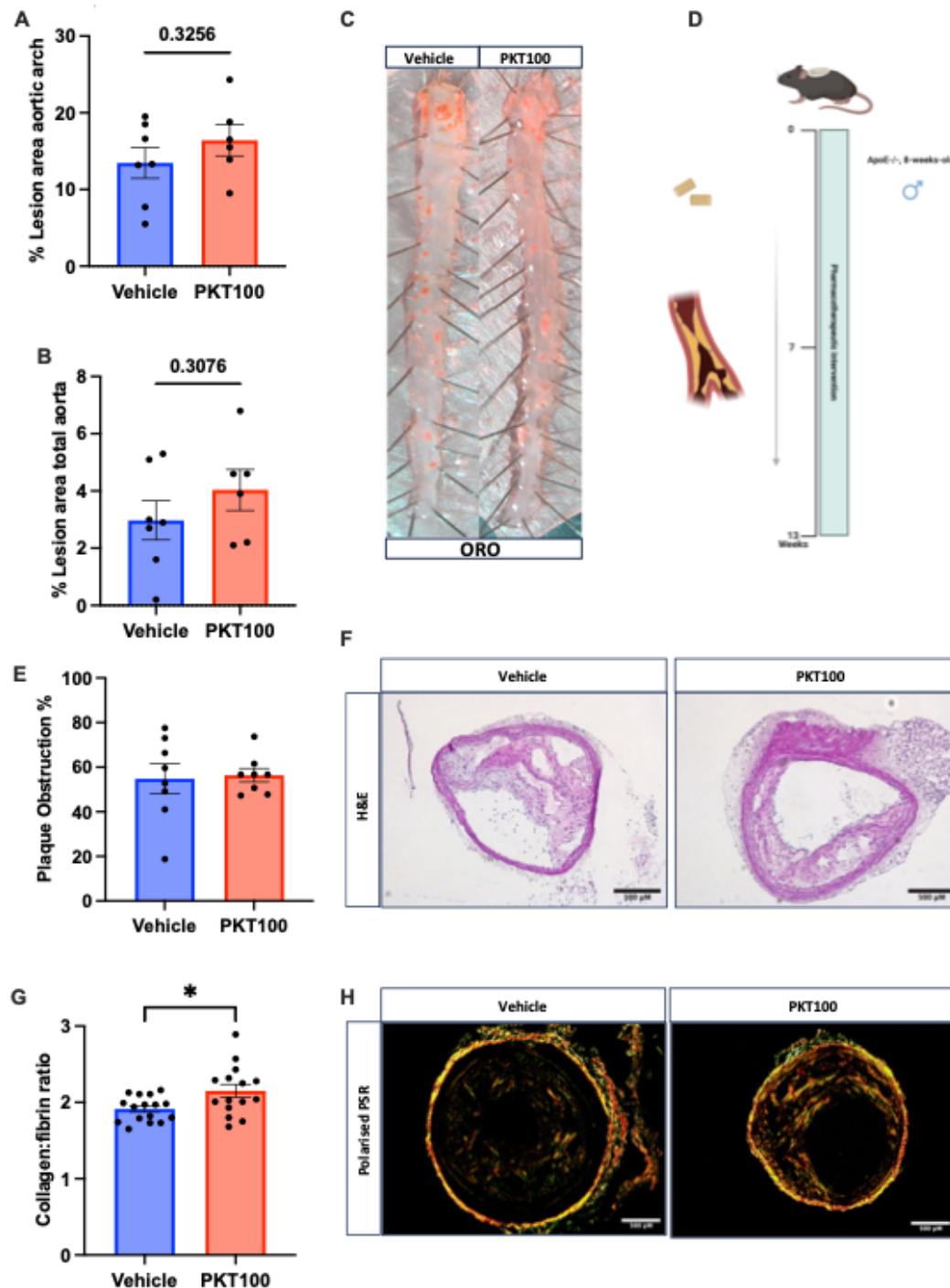


Figure 4. Plaque volume and progression in the aorta remote from the tandem stenosis. **A)** Quantification of atherosclerotic burden in the aortic arch with Oil Red O (vehicle, n=7, PKT100, n=6). **B)** Quantification of the atherosclerotic burden in the abdominal aorta (vehicle, n=7, PKT100, n=6). **C)** Representative images of atherosclerotic plaque in whole aorta from PKT100 and vehicle-treated mice. **D)** Schematic of the protocol overview and drug delivery procedure. **E)** Quantification of atherosclerotic plaque size in a stable atherosclerosis model (vehicle, n=8, PKT100, n=8). **F)** Representative images of stable segments in the brachiocephalic artery from PKT100 and vehicle-treated mice. **G)** Quantification of collagen-rich extracellular matrix in unstable atherosclerotic plaque segments (vehicle, n=16, PKT100, n=15). **H)** Representative images of collagen I and III expression under polarised lens. Values are expressed as mean $\pm$ SEM. *P<0.05.

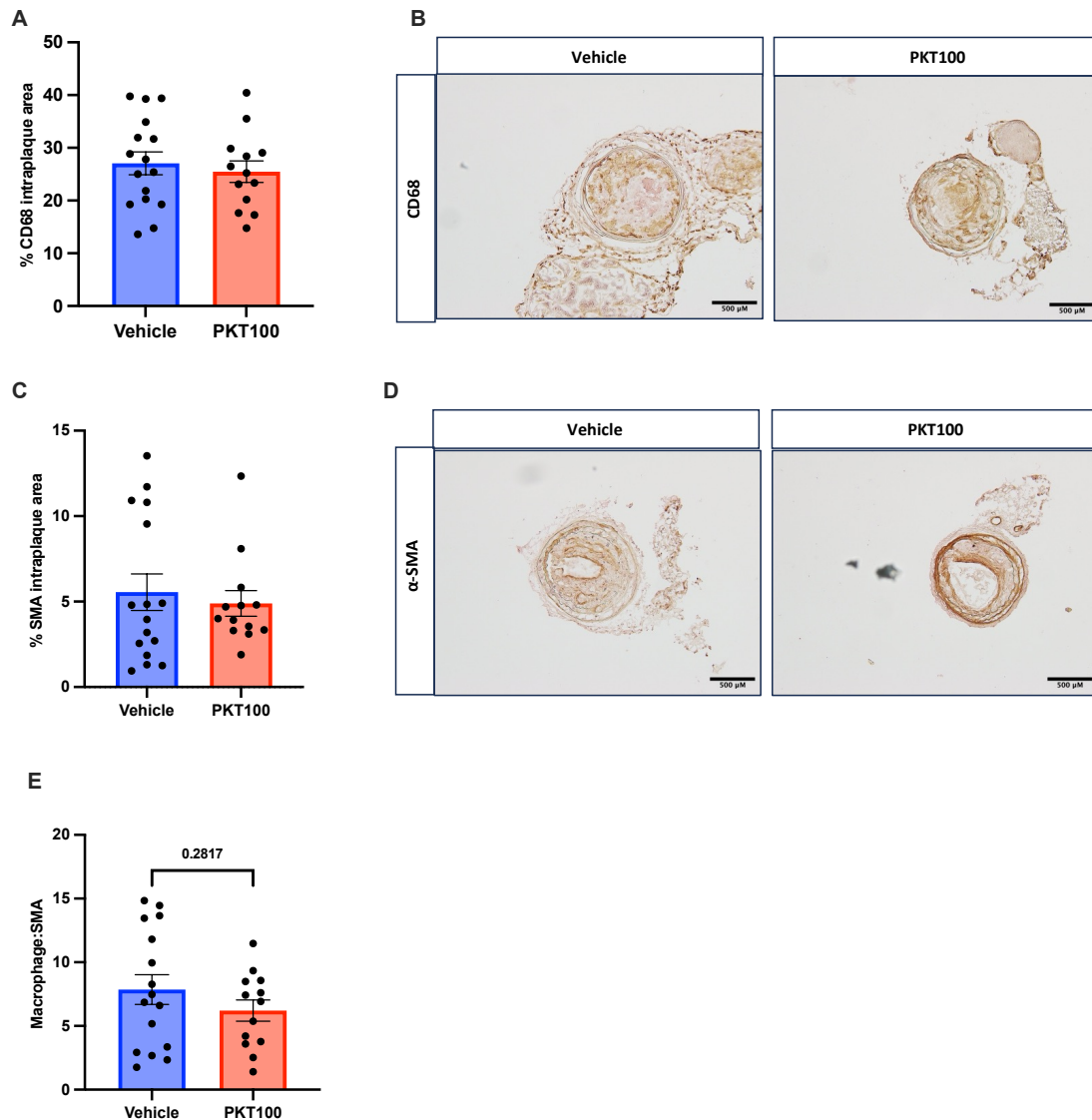


Figure 5. PKT100 is not associated with a reduction in macrophage infiltration in atherosclerotic lesions. A) Quantification of macrophage infiltration in unstable plaque segments measured by CD68⁺ regions (**vehicle, n=16, PKT100, n=13**). **B)** Representative image of CD68⁺ macrophage stain in unstable atherosclerotic plaque segments from PKT100 and vehicle-treated mice. **C)** Quantification of the SMCs in unstable atherosclerotic plaque segments (**vehicle, n=16, PKT100, n=13**). **D)** Representative image of α-SMA stain in unstable atherosclerotic plaque segments from PKT100 and vehicle treated mice **E)** Quantification of the ratio between macrophage and SMCs in unstable atherosclerotic plaque segments (**vehicle, n=16, PKT100, n=13**). Values are expressed as mean ± SEM.

Chapter 5

Antagonism of the P2X7R with PKT100 ameliorates diastolic dysfunction and fibrosis in a mouse model of obesity-induced cardiometabolic syndrome

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Abstract

Approximately 50% of heart failure presentations occur in patients with preserved left ventricular ejection fraction. Current pharmacotherapeutic strategies for these patients are limited, although some promise has been seen with sodium-glucose cotransporter-2 inhibitors. While modulation of metabolic risk factors such as obesity, hypertension, hyperlipidemia, and glucose intolerance are essential in disease management, other unexplored mechanisms might contribute to the pathogenesis of heart failure with preserved ejection fraction (HFpEF). The P2X purinoceptor receptor (P2X7R) has been proposed as a therapeutic target, given its critical role in the downstream modulation of cytokines interleukin-1 beta (IL-1 β) and interleukin-18 (IL-18) release. To explore the potential efficacy of P2X7R antagonism in diastolic dysfunction, a murine model of obesity was employed to exacerbate pathways linked with metabolic-induced inflammation. C57BL6/J male mice received a 40% high-fat diet for 24 weeks. Sixteen weeks after high-fat diet commencement, mice had implanted subcutaneously a micro-osmotic minipump delivering either PKT100 or vehicle for an additional 8 weeks. Mice treated with the P2X7R antagonist PKT100 demonstrated protection against diastolic dysfunction and improved myocardial deformation measured with two-dimensional echocardiography. P2X7R antagonism had no effect on lean body mass or metabolic parameters but was associated with a 0.52 reduction in myocardial fibrosis compared to vehicle control. Bulk gene expression analysis from heart tissue showed significant reductions in inflammatory and fibrotic markers in mice treated with PKT100. Exploration of a publicly available dataset from patients with dilated cardiomyopathy (DCM) showed moderate P2X7R expression across cardiac macrophages, whereas P2X7R expression in cardiac fibroblasts was negligible. However, a strong paracrine relationship was determined between macrophages and fibroblasts, which was heightened in the setting of DCM. Further analysis indicated an increased pro-inflammatory environment in DCM participants, with less efficient ATP to adenosine conversion.

Introduction

In the last decade, there has been a shift towards a greater reduction in age-standardised mortality linked with atherosclerotic cardiovascular disease (ASCVD) which has intersected with the concomitant rise in heart failure with preserved ejection fraction (HFpEF). (1) The increasing prevalence of HFpEF, above heart failure (HF) with reduced ejection fraction (HFrEF), is at least partially attributed to the global surge of obesity, insulin resistance, and hypertension (2,3), in addition to an ageing population. These modifiable risk factors play a pivotal role in the presentation of diseases. (4) HFpEF is a syndrome with considerable clinical heterogeneity, reflecting that its manifestation does not arise from a single pathogenesis. (1,5,6) This has posed significant obstacles in the

advancements of diagnostic and therapeutic strategies for the condition, which remains a substantial unmet need in this cohort. While the modifiable risk factors are similar to ASCVD cohorts, (7) the population affected varies and mostly includes elderly, post-menopausal women. (8) Thus, a persistent sex disparity remains in the treatment of patients with HF, particularly given the limited efficacy and targeted available therapeutics for the treatment of HFpEF. (9–11)

For the first time, recent human trials in cohorts with HFpEF have shown promise for this challenging population. Notably, the sodium-glucose cotransporter 2 inhibitor (SGLT2), Empagliflozin, used in the management of type 2 diabetes mellitus, demonstrated some benefit on composite primary outcomes, which included reduction in cardiovascular mortality and hospitalisation for HF. (12) Although, the benefit was predominantly driven by hospitalisation for HF rather than mortality. (2) These results were replicated with Dapagliflozin, which also showed benefits on primary outcomes, primarily driven by reduced hospitalisation. (12) The beneficial effects of SGLT2 inhibitors on heart failure endpoints in HFrEF and HFpEF are not fully understood. While some contribution is associated with improvements in the systemic metabolic milieu and blood pressure regulation, (2,12) SGLT2 inhibitors - are hypothesised to reduced pro-inflammatory-oxidative pathways and protein kinase G α oxidation in HFpEF. (13) However, there remains considerable potential for further improvements in addressing the effect of inflammation in HFpEF. (14,15)

The P2X purinoceptor 7 (P2X7R) has garnered significant attention as an upstream modulator of interleukin-1 beta (IL-1 β) and interleukin-18 (IL-18) release. (16–18) The receptor is activated in response to extracellular adenosine-5'-triphosphate (ATP), where it promotes downstream activation of inflammasome complexes that initiate proteolytic cleavage of stored cytosolic precursor cytokines and the N-terminal fragment of gasdermin-D. (19,19,20) This mechanisms has been shown to facilitate the release of mature bioactive cytokines, promoting immune-mediated crosstalk with organ-specific cell populations and the recruitment of exogenous immune cells. (19,21), Previous studies with the P2X7R antagonist, Brilliant Blue G in a mouse transaortic constriction model showed that Brilliant Blue G preserved systolic heart function and reduced interstitial fibrosis. (22) Compared to direct suppression of cytokine activity with monoclonal antibodies such as Canakinumab, (23–26) targeting the P2X7R offers a more nuanced approach, reducing inflammation associated with sterile cell injury and apoptosis. (16,17,27) Thus, substantial literature supports the therapeutic potential of a P2X7R antagonist in the treatment of HFpEF.

This study aims to address this critical gap by evaluating whether a P2X7R antagonists, PKT100 delivered systemically through micro-osmotic pump diffusion can protect against obesity-induced diastolic dysfunction in a preclinical mouse model. Obese mice treated with PKT100 showed protection against diastolic dysfunction and measures of myocardial stiffness compared to the control group. This was accompanied by reductions in interstitial fibrosis, independent of any change in lean body weight and metabolic parameters. Treatment with PKT100 was also associated with significant reduction in expression of inflammatory genes. Taken together,

these results provide preliminary evidence that modulation of the P2X7R with PKT100 may be beneficial as a therapeutic strategy in HFpEF.

Methods

Animal maintenance

All animal experiments adhered to the ethical standards and were conducted according to the Australian National Health and Medical Research Council (NHMRC) guidelines and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The Animal Ethics Committees at the University of Sydney (Australia) and Northern Local Health Districts (Australia) approved all procedures and experiments, under approval number 2022/2120. The well-being of the mice was prioritised throughout the study. Mice were group-housed and maintained on a fortified chow diet (SF00-100, Specialty Feeds, Australia) until the commencement of the study at the Kearns Facility at the Kolling Institute of Medical Research (Australia). Acidified water was provided *ad libitum*. Mice were housed in a 12-hour light-dark cycle, and the temperature was maintained at 21°C, with relative humidity between 45-60%. Seven days before the commencement of the study, mice were acclimised in the holding room to minimise potential stress.

Murine model of metabolic-induced cardiac dysfunction

Eight-week-old male C57BL6/J mice were sourced from the Kearns breeding facility (Australia). Mice were transitioned from the fortified chow diet to a semi-pure high-fat diet, where 42% of the energy intake was derived from lipids (SF04-001, Specialty Feeds, Australia). Mice remained on this diet for a total of 24 weeks with the aim to induce a cardiometabolic phenotype. After 16 weeks on the high-fat diet, under isoflurane, a lateral cut was made in the interscapular region and undermined to create a subcutaneous pocket for micro-osmotic minipump (Alzet model 1004 0.16 µL per hour for 28 days, Durect Corporation Germany) inserted to deliver 0.2 mg/kg/day of the P2X7R antagonist PKT100, or vehicle for 8 weeks. The osmotic minipump was replaced once to ensure constant delivery of PKT100 for 8 weeks. Mice were euthanised under isoflurane anesthesia. Blood was collected from the inferior vena cava using a heparinised 24-gauge needle (Sigma-Aldrich, USA), and the heart was removed and weighed, with the left ventricle dissected for downstream histology and molecular biology. For histological analysis, the left ventricle was isolated from the atria and right ventricle and embedded in optimal cutting temperature (Sakura Finetek, USA) and set on dry ice. The average weight gained within the 24-week timeframe in the study was 1.6 times the commencing bodyweight. Mice were excluded if they failed to demonstrate a 1.6-fold increase from baseline weight. Although no obesity criterion is established for mice, the literature indicates that mice bodyweight increases 1.3-fold on a chow diet between 12 to 36 weeks old

In vivo echocardiography

Mice were lightly anesthetised under 1-1.5% isoflurane to maintain a heart rate between 350 to 500 beats per minute (BPM), while respiration was maintained between 150 and 190 breaths per minute. Body temperature was retained at approximately 37°C, observed by a rectal thermometer (VisualSonics Inc, Canada), with mice

kept in the supine orientation on a heated platform. The fur was removed from the thoracic region to prepare mice for imaging acquisition by applying topical depilatory cream and ultrasound transducer gel to the chest. Transthoracic echocardiography was performed at baseline, 6-, 12-, 18-, and 24-weeks post-HFD induction with a Vevo3100 imaging system (Canada) equipped with a 40-MHz probe (MS400, Canada). The heart was imaged in the parasternal long- (PSLAX), and short-axis (PSAX) views to evaluate systolic function. From this angle, the heart was captured in B-mode and M-mode. In M-mode, the cursor was positioned after the papillary muscles. Image acquisition was used to derive ejection fraction and fractional shortening measures. Speckle-tracking (strain) echocardiography, such as global longitudinal strain (GLS), was measured from B-mode PSLAX and SAX images. Left ventricular diastolic function was assessed from a 4-chamber view in tissue and pulse wave Doppler modes. From this view, E velocity and A velocity, isovolumetric relaxation time, isovolumetric contraction time, ejection time, and no flow time were captured. Tissue Doppler images of the mitral valve were used to derive the E', A' and S' waves. Diastolic dysfunction was determined through the E/A ratio and E/e' parameters, where the E/A ratio was derived through the E velocity $\div$ A velocity and the E/e' was obtained by E velocity $\div$ e' wave velocity across the mitral valve.

Glycemia measures

Blood glucose concentration was measured at baseline and every two weeks via saphenous vein tail-prick (Freestyle, Abbott, USA). One week before the endpoint, mice underwent glucose tolerance tests to assess glucose metabolism. Mice were fasted for 24 hours before the baseline glucose concentration was recorded. At timepoint 0, mice were received with 1mg/g of glucose (Sigma-Aldrich) resuspended in sterile heated water via intraperitoneal administration. Blood glucose was monitored from the saphenous vein every 0-, 15-, 30-, 45-, 60-, 90-, and 120-minutes post intraperitoneal administration. Mice also underwent endpoint insulin tolerance tests and were fasted for 6 hours before their blood glucose assessment. 0.5-unit insulin concentration dissolved in saline (0.9% NaCl, Thermo Fisher Scientific, USA). Mice received the insulin via intraperitoneal route, and blood glucose was monitored as reported above.

Immunohistochemistry

The left ventricle was embedded unfixed in optimal cutting temperature and held at -80°C until cut. Sections were cut at 7 μ m on a NX70 CryoStar (Thermo Fisher Scientific) with the blade held at -20°C and the head at -15°C. After airdrying for 3 hours, sections were fixed with ice cold acetone (ASC reagent, $\geq$ 99.5% Sigma-Aldrich) and placed on dry ice. To ensure that slides were put into the -80°C completely dry, sections were allowed to air dry after acetone fixation for 30 minutes. After being removed from the -80C freezer, sections were allowed to come to room temperature for 1 hour. For assessment of interstitial collagen content, sections were stained with a modified picrosirius red stain (0.6% weight to volume fast red 80 dye (Sigma-Aldrich), reconstituted in 1.3% saturated aqueous picric acid (Sigma-Aldrich) optimised for frozen sections. Slides were incubated in Bouin's solution (Sigma-Aldrich) for 30 minutes at room temperature before incubating in PSR solution for 30 minutes at room temperature. Samples were differentiated with distilled water before being dehydrated in four

changes of ethanol, followed by four changes of xylene (Point of Care Diagnostics), and mounted with a histology-grade Eukitt resin (Sigma-Aldrich). All images were captured on a brightfield DM750 microscope with a 20x objective fitted to an Olympus DP22 camera (Olympus, Japan). For each slide, a total of 10 images were obtained of the tissue at random and without overlap to obtain a better estimation of the fibrosis across the tissue. All images were analysed using analysis software (open-source project ImageJ2). For the detection of pixels, all images were converted to 16-bit images and converted to binary to set a unanimous threshold. The total area of the images was used to derive the percentage of fibrosis for a given image. The averages of each of these images were summed and divided by the total quantity of images analysed for a sample.

Immunofluorescence

For the detection of cardiomyocyte size, heart sections preserved as described above were thawed at room temperature for 20 minutes. Sections were airdried at room temperature for 10 minutes. The area of interest was marked with a hydrophobic ReadyProbe Barrier Pap Pen (Thermo Fisher Scientific) and dried at room temperature for 60 seconds. Samples were promptly blocked with 2.5% BSA (Sigma-Aldrich) reconstituted in phosphate buffered saline (PBS, Thermo Fisher Scientific), for 30 minutes at room temperature in a hydration tray. Slides were washed two times with PBS for 3 minutes at room temperature. To identify cellular boundaries, wheat germ agglutinin (WGA) in the 555 wavelength was incubated for 30 minutes at room temperature in a lightproof hydration tray (Alexa Fluor-555, Thermo Fisher Scientific, 1:100 dilution in PBS). After this, samples were washed three times for three minutes with PBS and stained with NucBlue Fixed cell readyprobes reagent (Life technologies, USA) for 15 minutes at room temperature. Slides were washed as detailed above and mounted with fluorescent G-mount liquid (Thermo Fisher Scientific). The mounting liquid was allowed to dry for 30 minutes at room temperature. Slides were stored at 4°C in a lightproof box. Images were captured on an AxioImagerM1 microscope at 40 times magnification. Ten images were captured for each heart section to provide an estimated view of the entire myocardium. Images were carefully selected to ensure no overlaps. All 10 images were analysed. Ten cardiomyocytes were chosen in the transverse plane for assessment for each image. To evaluate cardiomyocyte hypertrophy, cardiomyocytes were outlined to acquire their size and width. Cardiomyocytes were averaged for each image and averaged again for each sample to gain an estimation of cardiomyocyte hypertrophy. All histological assessments were done manually and completed using Fiji image analysis software (open-source project ImageJ2).

RT-PCR Gene Expression Assessment

To assess transcriptional expression from heart samples from the left ventricle, heart samples were cut into smaller pieces and snap frozen. Total RNA was extracted from the left ventricular tissues with TRI Reagent (Sigma-Aldrich) as per the manufacturer's guidelines. The tissue was then subjected to standard RNA to cDNA conversion with superscript Vilo IV (Thermo Fisher Scientific). Following this, the expression of genes of interest was determined with PowerTrack SYBR Green Master Mix. Transcripts that were tested are as follows in mouse: IL1b (Forward: GCAACTGTTCTGAACTCAACT, Reverse: ATCTTTGGGGTCCGTCAACT), P2rx7 (Forward:

GACAAACAAAGTCACCCGGAT, Reverse: CGCTCACCAAAGCAAAGCTAAT), *Ccl2* (Forward: GCTGGAGAGCTACAAGAGGATCA, Reverse: CTCTCTCTTGAGCTTGGTGACAAA), *Adgre1* (Forward: TGACTCACCTTGTGGTCCTAA, Reverse: CTTCCCAGAATCCAGTCTTTCC), *Tgfb1* (Forward: CAACAATTCCTGGCGTTACC, Reverse: AGCCCTGTATTCCGTCCT), *Col1a1* (Forward: GCTCCTCTTAGGGGCCACT, Reverse: CCACGTCTCACCATTGGGG), *Col1a3* (Forward: CTGTAACATGGAAACTGGGGAAA, Reverse: CCATAGCTGAACTGAAAACCACC), *Mmp9* (Forward: AGACGACATAGACGGCATCC, Reverse: TCGGCTGTGGTTCAGTTGT). Genes were applied with a standard method in a 384-well plate using the QuantiStudio 12K Real-Time PCR System (Applied Biosystems, USA).

Protein Expression

Protein expression from the left ventricle of the mouse heart sample was evaluated from the same heart samples from which RNA was isolated. In short, the left ventricle was removed from the -80°C and placed into a new microcentrifuge tube (Eppendorf, Germany) on ice. Approximately 200 µL of 1% NP40 buffer (Thermo Fisher Scientific) reconstituted with a protease inhibitor cocktail and phosphatase inhibitor was added, and the sample was homogenised for 3 minutes off and on and placed on ice intermittently to prevent sample degradation. Samples were centrifuged at 10,000 *xg* for 10 minutes at 4°C to remove the non-digestible fibrotic components of the tissue. Samples were run on a Pierce bicinchoninic acid assay (Thermo Fisher Scientific) for protein quantification. A standard curve was included to interpolate the concentrations depending on absorbances to acquire a numerical concentration in micrograms per millilitre. Samples were reconstituted at a final concentration of 30 µg for in 40 µL. Samples were reconstituted with 4 µL reducing agent (Thermo Fisher Scientific) and 10 µL loading buffer (Thermo Fisher Scientific) and denatured at 75°C for 10 minutes before being transferred to ice. Proteins were loaded on a 4-12% NuPAGE Bis-Tris protein gel (ThermoFisher Scientific) and transferred in 1X 2-N-morpholino diluted in deionised water at 120 V for 2 hours (Thermo Fisher Scientific). After electrophoresis, proteins were transferred from the gel to a polyvinylidene fluoride membrane in transfer buffer (Tri-buffered saline 7.4 pH reconstituted with 20% methanol and 0.1% Tween 20 (Thermo Fisher Scientific)). Membranes were blocked for 1 hour at room temperature in PBS Intercept Blocking Buffer diluted 1:1 with PBS (LI-COR Biosciences, USA). Membranes were then incubated with 1:200 Rabbit polyclonal anti-mP2X7R (Proteintech, USA) and 1:1000 Mouse anti-β-actin antibody (Cell Signaling Technology, USA) overnight at 4°C. The membrane was washed three times for 5 minutes each in TBS with 0.1% Tween 20 and incubated with 1:10,000 secondary fluorescent antibodies targeted against the host in a PBS. The membrane was washed three times with TBS with Tween 20 for 5 minutes. A fourth wash was completed in TBS alone before being imaged on a ChemiDoc MP Imaging System (Bio-Rad Laboratories, USA). The quantification of protein expression was measured based on pixel intensity and expressed at the relative value of the corresponding housekeeper.

Single-cell RNA sequencing analysis from patients with DCM

A publicly accessible dataset from a recent publication was accessed to leverage the utility of single-cell sequencing data. (28) The purpose of this initial study was to provide a comprehensive analysis of the

transcriptional and cellular landscape in human heart failure in patients with dilated cardiomyopathy. (28) The initial study integrated data from single-cell nuclei and single-cell RNA sequencing to better capture cellular diversification in a cohort with heart failure. The ROBJ formatted files from the single cell sequenced samples were acquired for downstream evaluation. Five participants were included with dilated cardiomyopathy, whereas two healthy participants referred to as donors were utilised in the study. The ROBJ file, saved as a native R object contained the already processed Seurat object, filtered for mitochondrial reads less than 10% and RNA counts below 10,000. (28) The file showed doublet removal transformation and normalisation to fit a binominal distribution. To perform unsupervised clustering, the functions FindNeighbours and FindClusters were used. This identified eleven significant cell populations in the heart. Comparisons between cell populations and conditions were evaluated through dimensionality reduction with Uniform Manifold Approximation and Projection. For the assessment of gene expression differences between donor and patients, the 'find all markers' function in Seurat was used. Genes with an adjusted P values <0.05 were significantly different. Figures were generated with the 'DotPlot()' function. For paracrine analysis, CellChat (version 2.1.2) was used. The standard tutorial was used in accordance with the authors previous publication. (29)

Statistical Analysis

Data was analysed with the GraphPad Prism 10 statistical software package (GraphPad Software Inc, USA). Gene expression analysis was performed using the 2-DDCT method to detect fold differences relative to the defined comparison samples. All data is presented as mean $\pm$ SEM. Unless otherwise indicated, all data was analysed by a two-way t-test. A P value of <0.05 was considered statistically significant.

Results

Development of a mouse model of obesity-induced metabolic-driven diastolic dysfunction

Obesity is a crucial contributor to metabolic syndrome that promotes the pathogenesis of diastolic dysfunction in heart failure. (1,30,31) This study evaluated whether treatment with a P2X7R antagonist could rescue the early physiological and morphological changes associated with HFpEF. Mice were separated into two groups and fed a 40% HFD for 24 weeks. After 16-weeks HFD treatment, mice received either 0.2 mg/kg/day PKT100 or vehicle for an addition 8 weeks (**Figure 1A**). As shown in **Figure 1B**, mice doubled their body weight compared to baseline over 24 weeks, with no differences related to P2X7R antagonist PKT100 administration (49.0 ± 6.5 vs 49.4 ± 5.5 , $p=0.82$; $n=10-11$, **Figure 1B**). Next, mice were evaluated as to whether they developed hyperglycemia in this model. Baseline concentrations of non-fasted glucose were similar in the mice allocated to PKT100 compared to vehicle treatment (**Figure 1C**). Treatment with PKT100 was not associated with any change in non-fasted glucose levels after 24 weeks compared with control obese mice (8.5 ± 0.3 vs 8.9 ± 1 $n=10-11$, **Figure 1C**).

Early type 2 diabetes mellitus often presents with insulin resistance driven by lipotoxicity in skeletal muscle, activating oxidative stress and inflammatory pathways. (32) Mice in this model were evaluated as to whether they demonstrate early indications of insulin resistance as shown by a fasted glucose tolerance test. Both

treatment groups showed more than a two-fold increase in glucose concentration after intraperitoneal delivery of glucose (2.4-fold vs 2.1-fold, $p < 0.0001$, **Figure 1D**). After 120 minutes, glucose concentrations remained slightly elevated in both cohorts, consistent with glucose intolerance (**Figure 1D**). As shown in **Figure 1D**, PKT100 treatment was not associated with any change in glucose tolerance test compared to the vehicle ($AUC\ 49.69 \pm 7.02$ vs 50.84 ± 4.77 , $p = 0.749$, **Figure 1D**). These results confirm that 24 weeks of HFD was associated with insulin resistance and glucose intolerance, however, PKT100 treatment had no effects on these outcomes.

The accumulation of adipocytes is associated with the infiltration of immune cells, such as macrophages, which promotes a pro-inflammatory state. These deposits have demonstrated a paracrine relationship with other organs to promote dysregulation. To elucidate whether PKT100 influenced lean body mass, epididymal fat was measured and compared it to the final weight of mice. There was no discernible difference between the lean body mass of mice treated with PKT100 and the vehicle (28.2 ± 2.2 vs 24.8 ± 1.6 , $p = 0.23$, $n = 10-11$, **Figure 1E**). Liver weight was also measured as a downstream organ of PKT100 metabolism. No significant differences were observed in liver weight between PKT100 treated and vehicle mice (11.7 ± 1.0 vs 10.7 ± 1.3 , $p = 0.54$, **Figure 1F**). Similarly, PKT100 had no significant effect on kidney, pancreas, or skeletal muscle weight (**Figure G-I**). However, PKT100 was associated with a significant increase in the weight of the spleen (6.0 ± 0.32 vs 4.9 ± 0.32 , $p = 0.031$, $n = 10-11$, **Figure 1J**).

Treatment with PKT100 is associated with improved parameters of diastolic and myocardial function

Compared to HFrEF, HFpEF presents with preserved left ventricular ejection fraction (EF) with abnormal diastolic function. (33) Next, systolic function was measured after 24 weeks of HFD to confirm that systolic parameters remain preserved. Previous studies have confirmed that systolic function in mice is maintained around 60 – 80%, depending on the anesthetics utilised. (34) Mice demonstrated preserved left ventricular EF after 24 weeks of HFD (60.9 ± 0.4 vs 62.5 ± 1.0 , $p = 0.13$, $n = 10-11$, **Figure 2A**). Next, diastolic parameters were evaluated to assess whether PKT100 could protect against obesity-driven diastolic dysfunction. The mitral peak E/A wave velocity ratio, a measure of filling pressure into the left ventricle was measured. PKT100 was associated with a reduced E/A velocity ratio compared with vehicle-treated mice (1.3 ± 0.08 vs 1.6 ± 0.09 $p = 0.048$, **Figure 2B**). However, no significant differences were seen in the E/e' ratio, which combines the mitral peak E wave velocity, and the velocity attributed to the mitral annulus (-31.2 ± 1.9 vs -26.8 ± 0.7 , $p = 0.07$, **Figure 2C**). Under these circumstances, the assessment of myocardial deformation of the left ventricle was utilised, given that it has been proposed as a predictor of future cardiovascular performance and an early prognostic marker of cardiac dysfunction. (35,36) Treatment with PKT100 was associated with better preserved GLS compared to vehicle treatment (-18.1 ± 0.6 vs -15.8 ± 0.7 , $p = 0.02$; $n = 10-11$, **Figure 2D**). These results underscore that PKT100 may better preserve heart function associated with metabolic-driven diastolic dysfunction.

Immunomodulation with PKT100 is protective against myocardial interstitial fibrosis but not hypertrophy

Next, structure alterations in the myocardium instigated by metabolic-driven pathways were investigated. Particularly in HFpEF, physiological changes in diastolic function tend to coincide with interstitial collagen deposition, which promotes left ventricular stiffening and poor contractility. (6,30) To understand whether differences in function were associated with interstitial fibrosis deposition, interstitial fibrosis was measured with picrosirius red positive staining. Compared to vehicle treated mice, treatment with PKT100 resulted in a 0.52-fold reduction in interstitial fibrosis (2.1 ± 0.4 vs 1.1 ± 0.2 , $p=0.04$; $n=10-9$; **Figure 3A**). To confirm whether PKT100 reduced fibrotic markers at a transcriptional level, the expression of *Col1a1*, *Col3a1*, and *Tgfb1* was evaluated. Mice treated with PKT100 exhibited a 0.58-fold reduction in the transcriptional expression of *Col1a1* (1.7 ± 0.2 -fold change vs 1.0 ± 0.09 -fold change, $p=0.002$, $n=10-11$, **Figure 3B**). Comparatively, treatment with PKT100 did not result in significant reductions in *Col3a1* despite a notable trend (1.3 ± 0.1 -fold change vs 1.7 ± 0.2 -fold change, $p=0.1$ $n=10-9$, **Figure 3C**). Previous studies have determined the critical role of *Tgfb1* in downstream modulation of pro-fibrotic pathways. (31,37,38) Mice treated with PKT100 showed a 27% reduction in *Tgfb1* transcriptional expression compared to the vehicle-treated group (0.51 ± 0.03 -fold change vs 0.7 ± 0.06 -fold change, $p=0.029$, $n=10-9$, **Figure 3D**). The expression of metalloproteinases, such as *Mmp9* was measured given the strong association with the severity of HF and extracellular matrix remodelling (31,39,40). No significant differences were observed in response to PKT100 compared with vehicle control (0.58 ± 0.1 -fold change vs 0.7 ± 0.1 -fold change, $p=0.029$, $n=10-9$, **Figure 3E**). Overall, these results support a potential anti-fibrotic role of PKT100 in obesity-driven diastolic dysfunction.

Cardiac hypertrophy plays a vital role in the pathogenesis of HFpEF (41), which is proposed to be driven by systemic inflammation and via a cyclic guanosine monophosphate and protein kinase G driven pathway. (42) There were no observable differences in left ventricle weights when normalised to tibia length between PKT100-treated mice and vehicle-treated control mice (6.7 ± 0.2 vs 6.3 ± 0.3 , $p=0.17$; $n=10-9$, **Figure 3F**). Histological assessment of cardiomyocyte mass and width was performed with cardiomyocytes identified in the transverse plane by fluorescent-bound lectin WGA. No differences in cardiomyocyte mass or width were revealed between groups (**Figure 3F-G**). These results indicate that P2X7R inhibition with PKT100 does not affect concentric hypertrophy.

PKT100 reduces the cellular inflammatory transcriptional profile of the myocardium

Next, the contribution of inflammatory-driven pathways in the pathogenesis of interstitial fibrosis associated with metabolic stress was examined. Bulk qPCR on mouse hearts from PKT100 or vehicle-treated mice was used to evaluate genes affected by downstream activation of inflammatory pathways. The expression of *IL1b* was first assessed since its maturation and release are linked with the activation of the P2X7R. It was hypothesised that PKT100 blockade of the P2X7R would protect against the continuous transcriptional expression of the *IL1b* gene. Indeed, PKT100 treatment resulted in 37.5% reduction in myocardial *IL1b* expression compared to the vehicle group (1.5 ± 0.1 vs 2.4 ± 0.4 , $p=0.048$, $n=10-9$; **Figure 4A**). However, there was no discernible difference in the

expression of the P2X7R between groups (0.63 ± 0.04 vs 0.58 ± 0.05 , $p = 0.49$ $n=10-9$; **Figure 4B**). To validate this result at a protein level, bulk protein was isolated from the left ventricle of mice to detect the protein expression of the P2X7R. Treatment with PKT100 had no significant effect on protein expression of the P2X7R (0.98 ± 0.02 vs 0.95 ± 0.05 , $p = 0.45$, $n = 4$; **Figure 4B**). This data confirms that PKT100 treatment in mice under metabolic stress reduces myocardial *IL1b* expression but does not affect the levels of P2X7R protein expression, indicating downstream modulation of *IL1b* release by PKT100.

Previous literature has pointed to a ubiquitous and multifaceted role for the P2X7R in several different cell types. Despite this, its expression and function are most abundant in myeloid-presenting cells such as macrophages and monocytes. Murine C-C Motif Chemokine Ligand 2 or *Ccl2* is shown to direct monocytes from the circulation to sites of inflammation and aid to macrophage differentiation. (43,44) Under these assumptions, it was hypothesised that bulk transcription levels of murine *Ccl2* would be downregulated by PKT100 treatment, indicating a reduction in macrophage infiltration. Mice treated with PKT100 showed a 33.3% reduction in *Ccl2* expression compared to the vehicle group (1.0 ± 0.08 vs 1.5 ± 0.1 , $p = 0.025$, $n=10-9$, **Figure 4C**). The marker *Adgre1* in mice distinguishes resident cardiac macrophages as opposed to the bone marrow derived infiltrating macrophages. It has been shown that after myocardial infarction the number of resident macrophages marked by *Adgre1* expression decreases, while macrophages from the bone marrow increase. (45) It was postulated that PKT100 downregulation of *Ccl2* expression might reduce *Adgre1* expression in bulk qPCR. PKT100 treated mice demonstrated a 27.2% reduction in *Adgre1* expression compared to vehicle treatment ($1.6.0 \pm 0.2$ vs 2.2 ± 0.2 , $p = 0.024$ $n=10-9$; **Figure 4D**). These results support that pharmacological modulation of the P2X7R may reduce macrophage expression in the heart in obesity-driven diastolic dysfunction, although this final point requires further evaluation.

The P2X7R is endogenously expressed in cardiac macrophages from patients with DCM, which demonstrate increased crosstalk with fibroblasts

Given the possible contribution of PKT100 in mitigating interstitial fibrosis associated with substantial metabolic risk, it was hypothesised whether its therapeutic benefit might be modulated via an immunomodulatory mechanism. Given the absence of a publicly available dataset for patients with HFpEF, a single-cell dataset from patients with dilated cardiomyopathy (**DCM, n=5**) and Donors (**Donor, n=2**). (28) This dataset encompassed all cell populations obtained through whole-cell sequencing, except for cardiomyocytes, which are excluded due to their size. Integration of patient samples resulted in 22,282 cells, clustered into 11 cell populations (**Figure 5A**). From these eleven clusters, fibroblasts represented the highest proportion of cells (**Donor 54.6% vs DCM 44.9%**), followed by endothelial cells (**Donor 25.2% vs DCM 22.4%**), then pericytes (**Donor 8.3% vs DCM 12.5%**), smooth muscle cells (**Donor 2.3% vs DCM 1.6%**), macrophages (**Donor 5.2% vs DCM 9.1%**), T cells (**Donor 2.2% vs DCM 5.6%**), and monocytes (**Donor 1.4% vs DCM 1.4%**). After filtering, *P2RX7* expression was confirmed to be most highly expressed in macrophages and neuronal cells (**Figure 5B-C**). Although, there was no significant differences in expression of the *P2RX7* between DCM compared with Donor patients. The expression of their downstream

markers of P2X7R-mediated inflammation were also explored. Expression of downstream targets such as, the *NLRP3*, *CASP1*, and *IL1B* were predicted to be upregulated in DCM (**Figure 5D**). Compared to macrophages, the expression of P2X7R was negatable in fibroblasts (**AVE intensity** 0.111 vs 0.004, macrophages vs fibroblasts, **Figure 5C**). These results suggest that PKT100 may mediate its beneficial effects on myocardial fibrosis and diastolic function through macrophages, indicating a paracrine relationship between immune and other cardiac subsets.

To address this, CellChat analysis was employed to predict the intracellular communications between cells. In both Donor and DCM cells, the communication network between fibroblasts and macrophages was predicted to be strong (**Figure 5E**). This indicates the biological relevance of the macrophage-fibroblast axis in the heart, both in healthy and diseased states. However, when comparing the number of fibroblast interactions between DCM and Donor cells, fibroblast interactions were increased across cell types, including macrophages (**Figure 5F**). Further analysis of the communication network showed a greater importance of pathways related to inflammation, apoptosis, and extracellular matrix organisation in DCM cells (**Figure 5G**). Notably, adenosine signalling was predicted to be more active in Donor patients compared to the DCM cohort, where CD39 signalling also had greater influence in Donor cells (**Figure 5G**). CD39 has been previously demonstrated to have an important role in the conversion of extracellular ATP to adenosine. (46,47) The conversion of ATP into adenosine has been shown to trigger an immunological switch from ATP-dependent inflammation towards an anti-inflammatory state. (47) This evidence implies less efficient ATP to adenosine conversion in DCM patients, marking a potential upregulation in pro-inflammatory pathways, such as chemokine family, macrophage migration inhibitory factor and TNF-related apoptosis-inducing ligand (**Figure 5G**). This highlights a potential immunomodulatory mechanism associated with DCM pathogenesis, contributing to an observed pro-inflammatory environment.

Discussion

In this study, using an obesity-induced murine model of HFpEF, it was shown for the first time that direct inhibition of P2X7R with PKT100 could protect against metabolic-driven myocardial dysfunction. PKT100 treatment resulted in preserved diastolic function, and protection against interstitial fibrosis deposition. This was demonstrated independently of an improvement in metabolic parameters. Bulk transcriptional analysis of hearts from mice treated with PKT100 showed lower expression of inflammatory genes such as *IL1b* and *Ccl2*. This corresponded with a decrease in fibrotic markers *Tgfb1* and *Col1a1*. To understand whether this response could be immune modulated, a publicly available single-cell dataset was used to explore macrophage-fibroblast crosstalk in patients with DCM. *P2RX7* expression was primarily found in macrophages and neurons in the heart, while fibroblast expression was negligible. Macrophages from DCM patients heart cells were predicted to have increased interactions with fibroblasts, although the strength of interaction was also predicted to be important in Donors. Furthermore, evaluation of communication networks showed that adenosine signalling had a greater influence in Donor cells, which included CD39 expression. These pathways mark the conversion of ATP into

adenosine, a potential immunological switch from ATP-dependent inflammation towards an anti-inflammatory state. These signalling events were reduced in DCM cells, which may reveal heightened inflammatory burden in patients in response to extracellular ATP. This may point to the therapeutic potential of a P2X7R antagonist in HF.

Previous studies have evaluated the P2X7R in the setting of HFrEF to understand its potential role in attenuating inflammation associated with maladaptive remodelling. In murine studies, using Brilliant Blue G, a nonselective P2X7R antagonist, diabetic mice demonstrated benefits on cardiovascular outcomes after ischemia-reperfusion. (48) However, the effects on diastolic parameters have previously not been characterised. This study showed that mice treated with PKT100 demonstrated preserved diastolic function, possibly related to a reduction in interstitial fibrosis. Unlike in ischemic heart disease, where fibrosis is laid in response to the loss of cardiomyocytes, fibrosis in HFpEF is deposited interstitium and results in the stiffening of the left ventricle during diastole. (49) While the mechanisms known to regulate these pathways are largely unknown, accumulating evidence supports the role of inflammation as a central modulator in the pathogenesis of HFpEF. (1,50,51) Evidence from metabolomics studies from participants with HFpEF and chest dyspnea showed several inflammation associated metabolites. (52) Particularly, participants with HFpEF demonstrated reduced resolvin D1, a critical anti-inflammatory mediator that reduces cytokine release in an angiotensin II infusion model. (52,53) Mice that received resolvin D1 also showed improved cardiac function and reduced fibrosis. (54) Comparatively, heightened prostaglandin and linoleic acid derivatives were associated with a greater burden of HFpEF. (52) In the context of inflammation, prostaglandin synthesis is mediated by the release of IL-1 β through COX-2. (54) Previous human studies have evaluated prostaglandin analogues in HFpEF and pulmonary hypertension; however, they were terminated due to poor participant recruitment. (55)

Human trials have increasingly focused on the metabolic risk factors associated with the predominant HFpEF phenotype, with a substantial emphasis on treating extracardiac parameters to improve patient outcomes. (2,12) SGLT2 inhibitors has shown promise in HFrEF and HFpEF, but this benefit is attenuated in those with ejection fraction above 60% compared to those with reduced ejection fraction. (2,56) While a reduction in body mass index and better glucose tolerance are proposed to be the primary drivers of this benefit, recent studies have pointed to the benefit of these compounds as anti-inflammatory agents. (57) Particularly, the NOD-like receptor protein 3 (NLRP3) inflammasome has been highlighted as a key pathogenic pathway in the development of HFpEF. (58) In a recent study, mice with metabolic-driven HFpEF treated with the NLRP3 inhibitor MCC950 showed preserved diastolic function and reductions in inflammatory cytokines. (58) These results largely mirror the research findings presented here, although MCC950 achieved steep reductions in cardiomyocyte hypertrophy. (58) However, since a chow 'healthy' control was not included, in this study, evidence of cardiomyocyte hypertrophy could not be determined. Moreover, studies using the SGLT2 inhibitor empagliflozin was shown to downregulation of NLRP3 expression in an ischemia-reperfusion injury, resulting in the subsequent reduction in pro-inflammatory cytokines. (59) While these benefits were initially hypothesised

to be through direct inhibition of NLRP3, it was later demonstrated that intracellular calcium was reduced and mediated the downregulation of NLRP3-mediated inflammation. (59) As an upstream modulator of the NLRP3 inflammasome, the P2X7R is hypothesised to offer greater therapeutic benefit against ATP-induced cytokine release. The activation of P2X7R, which promotes NLRP3 assembly, fails to undergo desensitisation in response to ATP due to a permanent C-cys anchor on the membrane. (60) This suggests that even under NLRP3 inhibition, the P2X7R continues to promote the formation of a macropore, which facilitates the entry of large molecules that promote pyroptosis via an NLRP3-dependent mechanism.

The emphasis on transcriptomic approaches at a single-cell level has been essential to characterising cellular diversification and differentiation across the myocardium. (28,61) While an in-depth transcriptional approach has not been applied to tissue from patients with HFpEF, transcriptomics data from patients with non-ischemic cardiomyopathies has provided insights into the mechanisms that impair myocardial function. Tissue biopsies from patients with HFpEF may enable better histological and molecular analysis to characterise these unique individuals. This study found that P2X7R was abundant on macrophages in the heart, as well as neurons. The expression of the *P2RX7* in macrophages was not unexpected, but instead confirms previous literature. (17,27,62) Furthermore, the expression of the *P2RX7* was also found in a neuron subcluster, although this was not a focus of this chapter and was not evaluated in depth. The expression of *P2RX7* in neurons remains controversial, with some reports of P2X7R expression only localised to the astrocytes and microglia. (63) Given the expression of P2X7R in cardiac macrophages, it was hypothesised whether macrophages show crosstalk with fibroblasts, which may explain the reduction in fibrosis shown after P2X7R antagonism. This mechanism was previously shown in heart failure monocytes. (64) Particularly, fibroblasts stimulation with supernatant from mutant monocytes resulted in a substantial increase in extracellular matrix proteins. (64) The predicted communication network between macrophages and fibroblasts across disease states was strong, highlighting the significance of this interaction in cardiovascular biology. Despite this, a greater interaction between fibroblasts and macrophages was predicted in DCM, hinting that these cell interactions may contribute to disease-related maladaptation. Previous studies have shown that macrophages secrete pro-fibrotic mediators such as TGF- β , interleukin-10, platelet-derived growth factor, and vascular endothelial growth factor that promote collagen synthesis through fibroblasts. (65,66) This data supports the immune-mediated crosstalk hypothesis in HF, although this mechanism remains elusive in the context of the P2X7R. Hence, future studies should aim to unravel these complex mechanisms to identify potential therapeutic targets in HFpEF.

Compared to other immunomodulatory therapies, such as those evaluated for the treatment of coronary artery disease, P2X7R antagonists demonstrate substantial advantages related to their more specific target away from host-defence pathways. (25,27,67–69) Particularly, the P2X7R is a crucial source of cytokine production and release, exclusively activated in response to tissue injury. (17,27,70,71) Under these circumstances, the release of cytokines from immune cells is considered sterile and outside the constraints of pathways that mediate response to infections. (72) In previous human trials with P2X7R antagonists, infection has not been a substantial

risk for these populations. (73–77) This has also been validated in P2X7R^{-/-} mice that have do not display increased susceptibility to infection. (78) Instead, the more common symptoms were reported to be gastrointestinal-related but only on the highest formulation. (73–75,77,79,80) However, human trials with P2X7R antagonists have yet to be largely successful, with the termination of studies in cohorts with rheumatoid arthritis and Crohn's Disease. (73,79) This study showed early evidence of efficacy but failed to demonstrate therapeutic benefit after 12 weeks of treatment. (73,81) However, a significant challenge in this population is demonstrating additional advantages alongside potent anti-inflammatory agents, such as methotrexate. Previous studies in mice have shown that while P2X7R antagonists and anti-inflammatories offer similar benefits, their combined efficacy is not cumulative. (82)

Lastly, there is an increasing recognition of the need for improved sub-phenotyping of patients with HFpEF. Notably, for the translation of the P2X7R antagonist for HFpEF, it may be appropriate to consider companion diagnostics that can identify populations most likely to respond to pharmacotherapeutics. This is particularly relevant for a population with clonal hemopoiesis of indeterminate potential who demonstrate heightened inflammatory burden due to a relatively small population of clonally expanding mutant immune cells. (14,14,83) Although causality has not been confirmed, the elevated levels of IL-1 β associated with this condition make it a potential target population that may have enhanced benefits in addition to obesity and metabolic syndrome. The possible application of ATP-induced IL-1 β in an ex-vivo assay may be helpful to stratify those who demonstrated heightened immune activation through a P2X7R-dependent pathway.

Limitations

While this study has several strengths, including the use of a novel P2X7R antagonist with translational potential that exhibits sufficient potency at both rodent and human P2X7R, the employment of echocardiography measures for heart function, and the incorporation of single-cell data from human datasets, it is not without its limitations. To start, whilst the echocardiography measures were consistent with the historical literature of mouse HFpEF models, healthy mice were not examined. Thus, the effect of P2X7R antagonism in was only examined in mice on a HFD. For this reason, also, no conclusions can be drawn about the severity of the model regarding its cardiac phenotype compared with non-obese control. Previous studies have shown substantial variability in inducing pathogenic alterations to the myocardium through metabolic insults. In a recent study published by Schiattarella et al., 2021, treatment with a 60% HFD for 16 weeks failed to induce substantial discrepancies in heart function parameters compared to standard chow control. (84) In a separate study, mice treated with the same HFD reported by Schiattarella but no N(G)-Nitro-L-arginine methyl ester (L-NAME) showed substantial evaluations in metabolic parameters such as glucose concentrations after 16 weeks. (31) Mice in this study demonstrated impairments in diastolic measures, as indicated by the E/e' ratio. (31) In both studies, running time was significantly impaired, likely associated with mouse weight rather than lung oedema. (31,84) In a third study chow-fed mice, mice receiving a 45% HFD for 20 weeks demonstrated substantial elevations in the diastolic parameters E/A and isovolumetric relaxation time. (85) Markers of fibrosis and inflammation were

also increased in their model compared to chow-fed mice, although the combination of angiotensin II and HFD contributed the greatest towards fibrosis development. (85) In accordance with this evidence and observations, future studies will include chow-fed mice to elucidate treatment severity.

Selection of mice to recapitulate the clinical manifestation of HFpEF

Discrepancies in the induction of animal models among different laboratory groups may be attributed to mouse background and acquisition facility. The C57BL/6N mouse, wild-type for the nicotinamide nucleotide transhydrogenase (*Nnt*) gene involved in the electron transfer across the mitochondrial membrane, may be more appropriate for modelling HFpEF than the C57BL/6J mouse. (86) Silencing of *Nnt* reduces NADPH and hydrogen peroxide release, providing protection against HFpEF development. (87) Recent studies underscore this critical pathway in the pathogenesis of HFpEF in C57BL/6N mice. (88,89) Particularly, supplementing heart NAD⁺ in a 'two-hit' model of HFpEF ameliorated diastolic dysfunction and hypertrophy. (88) Despite this, the C57BL/6J mouse, resulting from a spontaneous loss-of-function mutation in *Nnt*, has become the most widely available mouse for preclinical research. Previous studies, such as Schiattarella and Tong et al. 2021, have been stringent in using C57BL/6N mice. (84,88) Other papers, such as Ranjbarvaziri et al., 2024 have utilised the common C57BL/6J background but have opted for surgical induction of HFpEF in combination with an HFD. (31) Each presents distinct advantages and disadvantages and recapitulates varying aspects of the condition. To confirm the clinical translatability of this study findings for the treatment of HFpEF, the next study will use the C57BL/6N mouse to evaluate the efficacy of PKT100.

Many animal models of HFpEF have failed to recapitulate the clinical features of the condition. This is partly due to the unknown biological mechanisms that aggravates the progression of HFpEF, together with fundamental biological differences between humans and rodents. Current guidelines recognise HFpEF as a complex and heterogeneous condition with diverse pathogenic factors. Although metabolic syndrome is likely a major contributor to the pathogenesis of the condition, it is clear that HFpEF is not driven by a single cause. A recent review by Capone likened the development of HFpEF and its risk factors to the clinical manifestations of nonalcoholic steatohepatitis, underscoring the significant extracardiac component associated with the condition. (30) Comparatively, the efficacy of PKT100 in diastolic dysfunction was only evaluated. Theoretically, other organs should be assessed in the future to provide insights into their contribution to obesity-induced HFD, together with the role of the P2X7R in mitigating disease pathogenesis. This includes behavioural characteristics such as the assessment of exercise tolerance with grip tests and motor function. This can be combined with pulmonary capillary wedge pressure to discriminate better pulmonary oedema linked with HFpEF.

The recent focus on the development of animal models that better recapitulate the symptoms of HFpEF has resulted in several 'two-hit' models. (84,90,91) Particularly, the combination of L-NAME, a nitric oxide synthase inhibitor and HFD to mimic the hypertensive and metabolic disturbances in patients with HFpEF. (92) Other models, such as using low-dose streptozocin, which primarily affects the β -islets in the pancreas in combination

with an HFD, have also been used to mimic the clinical manifestations of diabetes-associated cardiomyopathy. (93,94) In a previously unpublished study, a combination of streptozocin and HFD was used to recapitulate the better clinical manifestations of diabetes-associated cardiomyopathy (data not shown). This model failed to demonstrate pathogenic changes in the myocardium above what HFD induces alone. Moreover, even at low doses, the extent of toxicity affected mouse adiposity and led to early mortality. Another model uses transaortic constriction to induce pressure-overload changes in the heart with an HFD. (90) In this model, mice demonstrate diastolic dysfunction and exercise intolerance. (90) However, in all these studies, including this study, female mice were excluded given the substantial complexities around using young female mice for cardiovascular-related studies. (15,31,84) A fourth model, considered the 'three-hit' model, includes the addition of reproductively senescent female mice to better mimic the demographic affected. HFpEF is far more common in older, post-menopausal women compared with older men who are primarily impacted by HFrEF. (9–11) For the clinical development of PKT100, future studies will aim to include a broader range of features, using L-NAME and HFD, together with reproductively senescent female mice, to improve the translatability of the findings.

The missing cohort in cardiovascular single-cell transcriptomics cohorts

Lastly, in this study, scRNA-seq dataset from a cohort with DCM compared to donors was utilised to evaluate pathways upregulated in the failing heart. While the clinical manifestations of DCM are linked with progressive dilation caused by fibrosis and myocardial remodelling, patients often present with non-ischemic reduced EF. (95) This starkly contrasts what is currently understood about the clinical manifestations of HFpEF. Particularly in earlier phases of the disease, patients present with preserved ejection fraction, although the volume driven out of the left ventricle is reduced due to substantial hypertrophy. (51) However, in both instances, HFpEF and DCM can present with current unknown etiologies and show progressive fibrosis, resulting in increased myocardial stiffening. (1,95) Thus, a scRNA-seq dataset from patients with DCM was utilised since no current datasets are available to unravel the transcriptional signature of the heart at a single-cell level. This is likely reflected by the limited invasiveness of HFpEF treatments, where biopsies and implantable devices are relatively rare.

Comparatively, heart samples were collected from patients at the time of left ventricular assist device removal. (28) Under these circumstances, patient biopsies were not conducted unnecessarily, with unrequired risk. These samples likely represent tissue that would have otherwise been discarded. Thus, despite discrepancies between the clinical manifestations of HFpEF and DCM, this dataset offers a substantial opportunity to evaluate cellular diversification in the failing heart. A recent study published as a preprint has conducted scRNA-seq on murine hearts from HFpEF and myocardial infarction models. The requirement from high-impact journals for data to be shared offers a great opportunity for unbiased discoveries. However, further experiments will incorporate single-cell nuclei isolation and sequencing to understand better the transcriptional signature in mice with HFpEF treated with PKT100.

Conclusion

This study assessed the therapeutic potential of a P2X7R antagonist, PKT100, in mitigating obesity-induced diastolic dysfunction. Compared to vehicle-treated mice, treatment with PKT100 preserved diastolic function and reduced markers associated with interstitial fibrosis deposition. P2X7R was found in abundance on a macrophage, where substantial crosstalk was demonstrated between fibroblasts and macrophage populations. Mice treated with PKT100 also showed significant reductions in inflammatory markers, highlighting the involvement of inflammation in obesity-driven diastolic dysfunction. While further research is required to elucidate the therapeutic role of P2X7R in HFpEF, these findings provide early data for the potential efficacy of PKT100 in attenuating inflammation associated with the pathogenesis of HFpEF. Future studies should consider incorporating murine models that better reflect the broader complexity and heterogeneity in HFpEF pathogenesis, including common morbidities such as hypertension, hyperlipidemia, and hyperglycemia.

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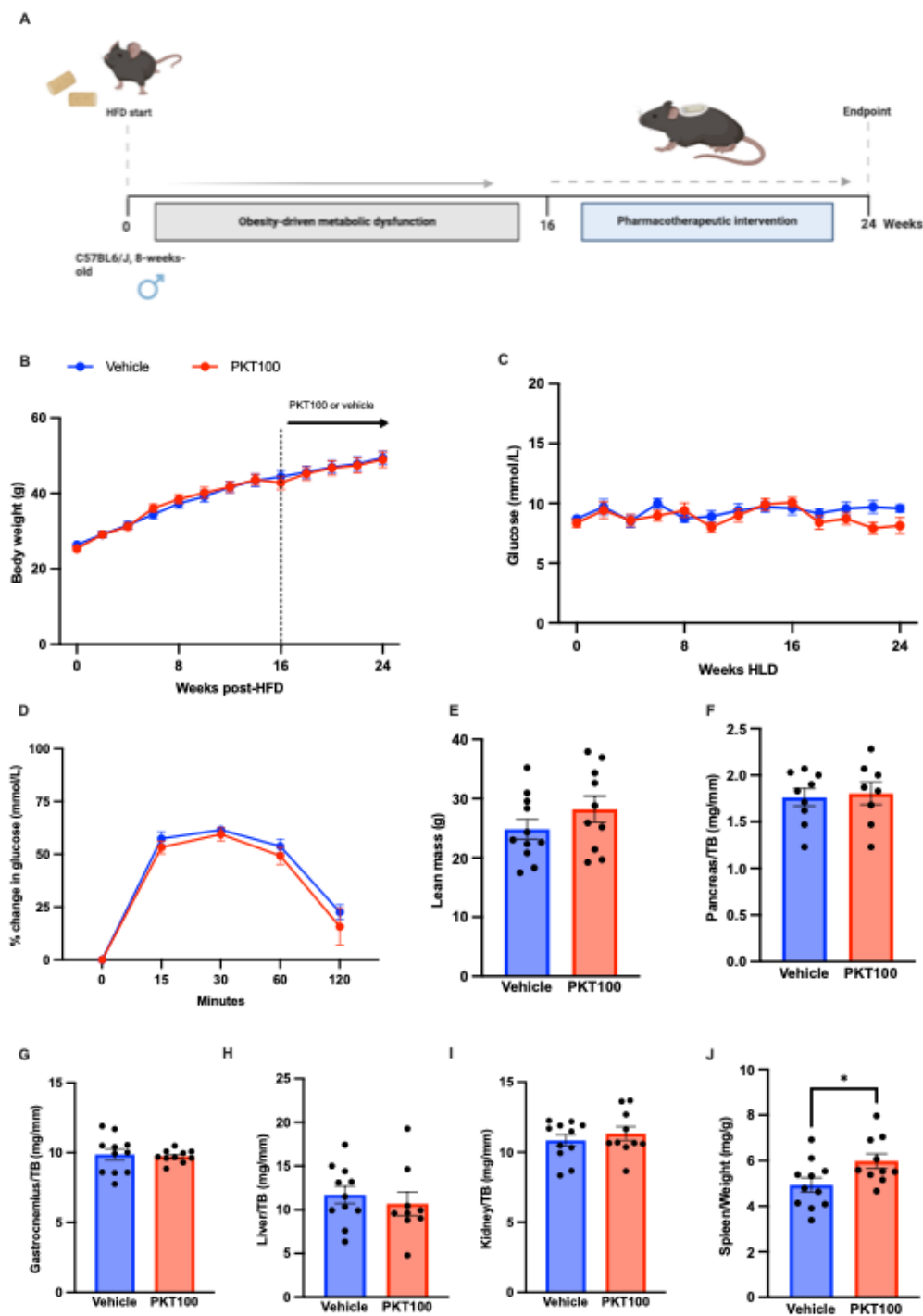


Figure 1. Twenty-four weeks of a HFD induces an obesity phenotype and metabolic disturbances that drives diastolic dysfunction. **A)** Schematic of the experimental design. **B)** Body weight of mice over 24 weeks (**vehicle**, n=11, **PKT100**, n=10). **C)** Blood glucose concentration measured over 24 weeks (**vehicle**, n=11, **PKT100**, n=10). **D)** Intraperitoneal glucose tolerance test reported as the percent change in glucose compared to the baseline glucose measured at the end of the protocol (**vehicle**, n=11, **PKT100**, n=10). **E)** Lean body mass reported as the ratio between terminal body weight and abdominal adiposity (**vehicle**, n=11, **PKT100**, n=10). **F-J)** Organ weights after 24 weeks on an HFD and 8 weeks of treatment with either PKT100 or vehicle (**vehicle**, n=11, **PKT100**, n=10). Data are presented as mean±SEM. *P<0.05, Created with Biorender.

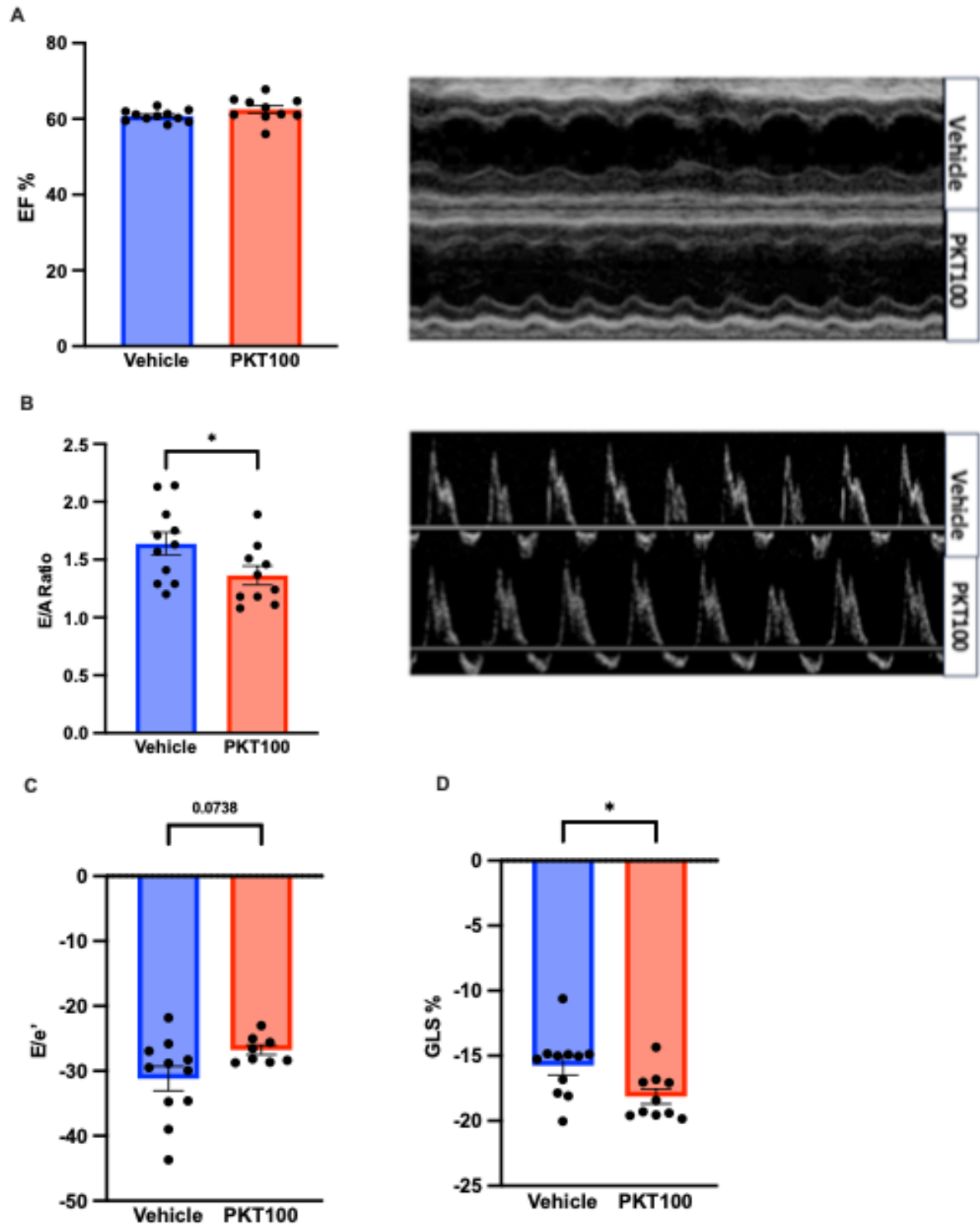


Figure 2. PKT100 improves cardiovascular function parameters associated with diastolic dysfunction. A) Echocardiography measures of ejection fraction measured in the PSLAX after 24 weeks of HFD and 8 weeks of drug treatment (**vehicle, n=11, PKT100, n=10**). **B)** Echocardiography measures of E/A wave velocity measured as the brightest point in pulse wave Doppler mode after 24 weeks of HFD and 8 weeks of drug treatment (**vehicle, n=11, PKT100, n=10**). **C)** Echocardiography measures of E/e' wave velocity measured at the mitral annulus in tissue Doppler mode after 24 weeks of HFD and 8 weeks of drug treatment (**vehicle, n=11, PKT100, n=8**). **D)** Left ventricular GLS measured with echocardiography (**vehicle, n=11, PKT100, n=10**) Data are presented as mean $\pm$ SEM. *P<0.05.

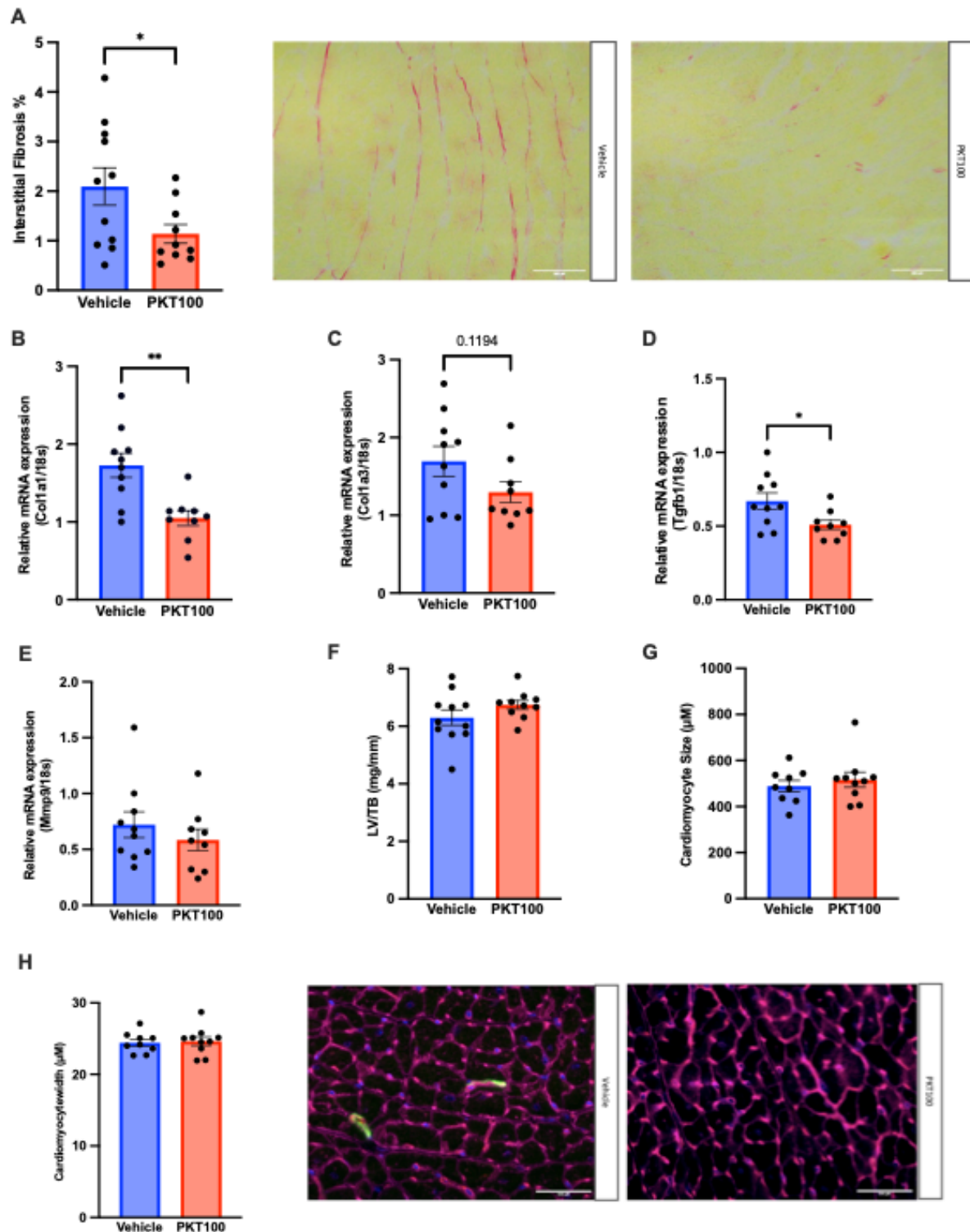


Figure 3. PKT100 ameliorates obesity-driven interstitial fibrosis. **A)** Quantification of myocardial interstitial fibrosis from the left ventricle measured with PSR (vehicle, n=11, PKT100, n=10). **B-D)** mRNA expression fold change in fibrosis markers **B)** *Col1a1*, **C)** *Col1a3* and **D)** *Tgfb1* (vehicle, n=10, PKT100, n=9). **E)** mRNA expression fold change in *Mmp9* (vehicle, n=10, PKT100, n=9). **F)** The ratio between left ventricle weight and tibia length (LV/TB) (vehicle, n=11, PKT100, n=10). **G-H)** Quantification of cardiomyocyte hypertrophy from the left ventricle with WGA (vehicle, n=9, PKT100, n=10). Data are presented as mean $\pm$ SEM. *P<0.05, ***P<0.005

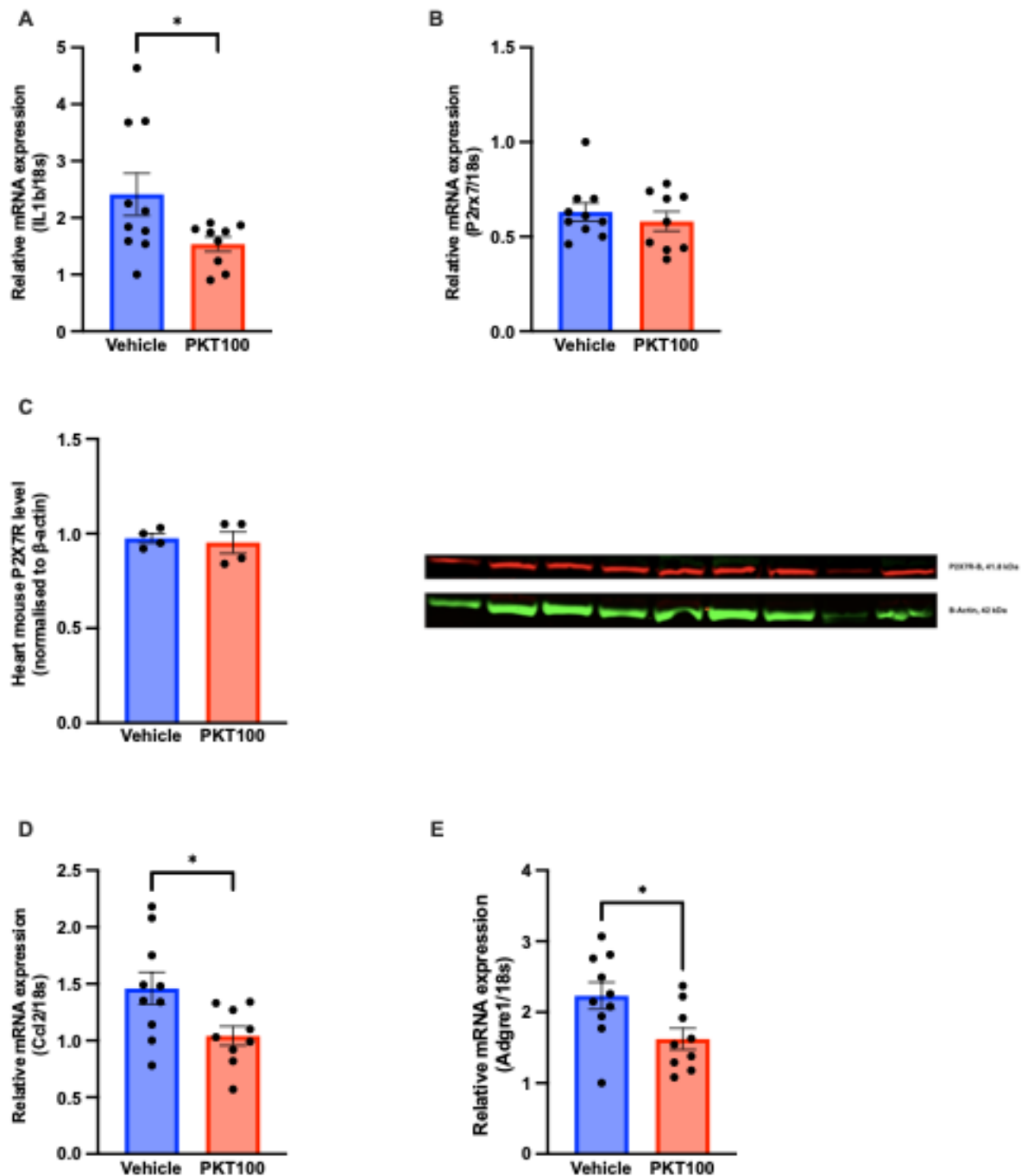


Figure 4. Treatment with PKT100 reduces inflammatory markers associated with myocardial immune activation. **A)** mRNA expression fold change in inflammatory marker *IL1b* (vehicle, n=10, PKT100, n=9). **B)** mRNA expression fold change in *P2rx7* (vehicle, n=10, PKT100, n=9). **C)** Representative western blot of P2X7R for validation of the transcriptional data (vehicle, n=4, PKT100, n=4). **D)** mRNA expression fold change in *Ccl2* (vehicle, n=10, PKT100, n=9). **E)** mRNA expression fold change in *Adgre1* (vehicle, n=10, PKT100, n=9). Data are presented as mean $\pm$ SEM. *P<0.05

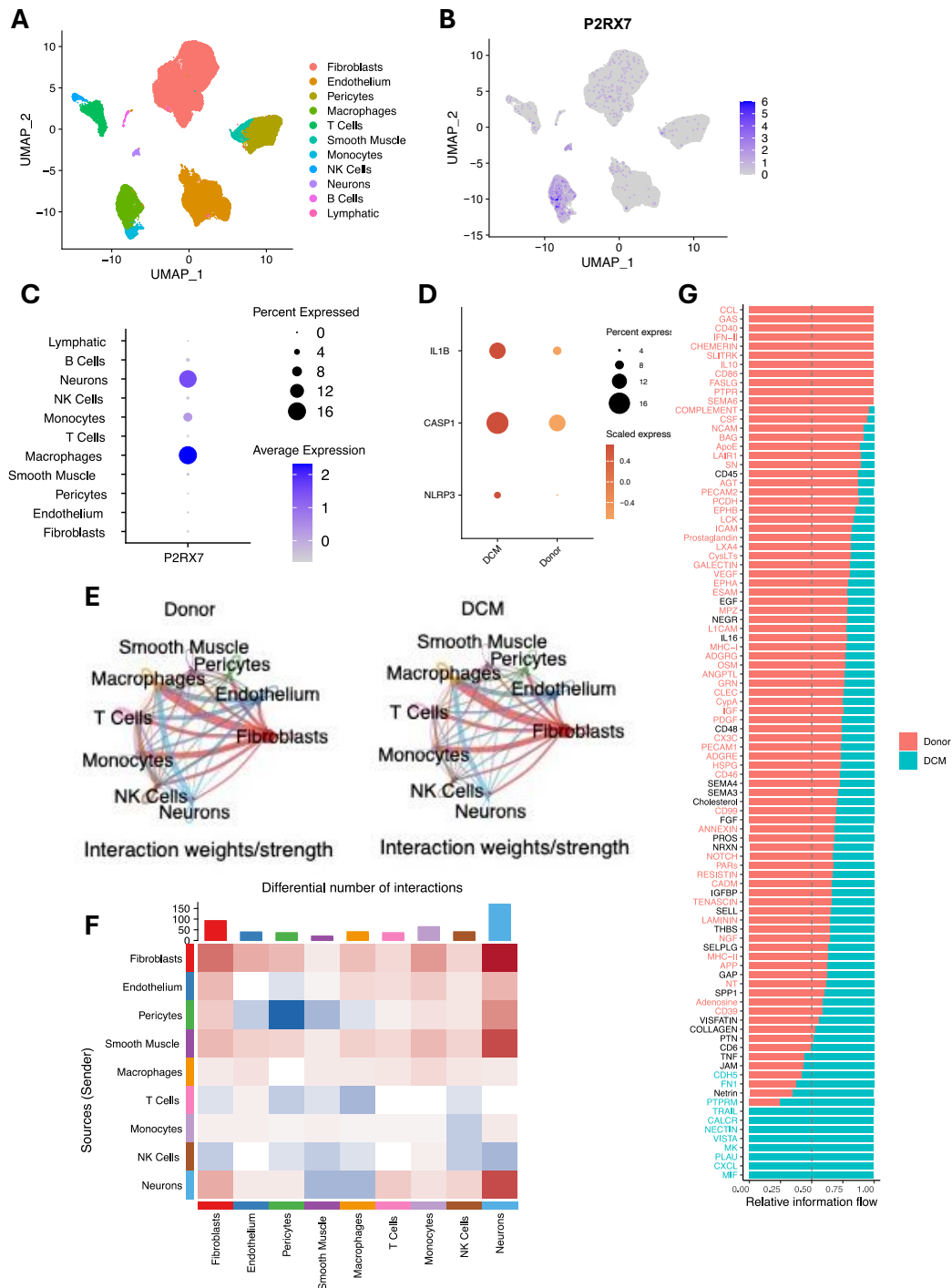


Figure 5. Single-cell analysis from the left ventricle of patients with dilated cardiomyopathy (DCM) reveals P2X7R in cardiac macrophages and increased incidences of macrophage-fibroblast crosstalk. A) Unsupervised Uniform Manifold Approximation and Projection (UMAP) clustering of 22,828 cells with integrated dataset split by cell types. **B)** UMAP of unsupervised clustering with subsets expressing the P2X7R underscored in blue pixels. **C)** Dot plot showing the expression of P2X7R across cell-types in the heart. **D)** Dot plot showing the expression of P2X7R downstream targets between disease states. **E)** Interaction weight and strength network between cells between disease states. **F)** Cumulative heatmap summarising the predicted number of interactions across cell types across disease states. **G)** Relative information flow predicts the strength of signalling pathways between disease states.

Chapter 6

Discovery and characterisation of potent small-molecule P2X7R antagonists for the treatment of persistent inflammation in coronary artery disease.

Elijah Genetzakis, Jayson Gilchrist, Andre McKenzie, Tung Nguyen, Sina Fathieh, James O'Brien Brown, Aditya Bhat, Eryn Werry, Michael Kassiou, Gemma A. Figtree

Abstract

Inflammation is now established as a central pathophysiological mediator of coronary artery disease (CAD). Its role as an effective therapeutic target is supported by evidence from the Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS), which showed the direct clinical benefit of interleukin 1 beta (IL-1 β) neutralisation in a cohort with a recent history of myocardial infarction (MI). However, clinical translation of these compounds has been hindered by increased susceptibility to bacterial sepsis. The P2X purinoceptor 7 (P2X7R) is a valid candidate due to its direct role in the maturation and release of cytokines in response to tissue injury via activation of the NOD-like receptor protein 3 (NLRP3) inflammasome. In this study, the pharmacological characterisation of novel cyanoguanidine-adamantane and adamantane amide hybrid P2X7R antagonists are reported. The cyanoguanidine series demonstrated improved potency compared to the first-generation adamantane benzamide antagonist PKT100. However, the success of the class was hindered by poor metabolic stability, making them inappropriate for clinical development. The synthesis of the adamantane amide series resulted in compounds with improved potency and metabolic stability. Based on the initial in vitro pharmacokinetics, these compounds have demonstrated early promise in the clinical development for CAD.

Introduction

Current strategies in the treatment of coronary artery disease (CAD) are focused on the management of traditional risk factors such as hypertension, hypercholesterolemia, and diabetes mellitus. (1,2) In the past three decades, this has resulted in a steady decline in the global incidence and mortality from CAD. (1,3) Despite this, mortality from cardiovascular disease (CVD) remains the most prevalent global burden. (1,4,5) The persistence in mortality from CVD may indicate potential biological mechanisms that remain unaccounted, despite secondary prevention efforts. Although decades of research that has pointed to the critical role of inflammation in the pathogenesis of CAD, only recently has this pathway been evaluated for cardiovascular indications. (6,7) The Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS) showed that interleukin-1 beta (IL-1 β) neutralisation could be beneficial against recurrent CVD events in a cohort with residual inflammatory following myocardial infarction (MI). (8) Canakinumab treatment in this cohort was associated with a 15% reduction in CVD events, independent of an effect of plasma lipid measures. (8) This finding represents a substantial advancement in identifying pathways beyond traditional risk contributing to CAD progression. Despite this, the increased susceptibility to fatal infections associated with Canakinumab underscores a critical requirement for safer and more effective pharmacotherapies that modulate inflammation in atherosclerosis. (8)

In atherosclerosis, the activation of the innate immune system in response to vascular inflammation resulting from the accumulation of low-density lipoprotein (LDL) promotes the earliest formation of atherosclerotic lesions. (9–11) While macrophages initially promote LDL clearance from vascular walls, this mechanism is eventually overwhelmed, leading to necroptosis and necrotic core formation. (12,13) At this point, atherosclerotic lesions adopts an unstable phenotype, where persistent biomechanical stress leads to vascular smooth muscle cell differentiation and the emergence of calcified atherosclerotic plaque. (14) Current risk stratification for CAD patients relies on the coronary artery calcium score (CACS), a crucial tool for cardiovascular risk prediction. The timely implementation of risk management strategies has been demonstrated to improve patient outcomes. (4,15) Although we are well placed in our current knowledge of what drives atherosclerotic development, recent epidemiological studies showed that the five modifiable risk factors, namely, hyperlipidemia, hypertension, obesity, diabetes mellitus, and smoking, account for only 58% of CVD incidences. (16) Under these circumstances, crucial aspects of the underlying biology remain unaddressed in CAD.

Upstream of IL-1 β release, the P2X purinoceptor receptor 7 (P2X7R) is a master regulator of sterile cytokine maturation and release. (17–20) At high concentrations (>100 μ M), adenosine-5'-triphosphate (ATP) acts at the receptor to elicit macropore formation in response to cellular injury. (17,21,22) This event evokes the assembly of the NOD-like receptor protein 3 (NLRP3) inflammasome that promotes caspase-1 activation. (23) Precursor cytokines, particularly pro-interleukin-1 beta (IL-1 β) and pro-interleukin-18 (IL-18) reserved in the cytoplasm, are cleaved by caspase-1 into their mature forms. (23–25) However, the release of these cytokines has been proposed to be mediated through two independent pathways and is dependent on the magnitude of the stimulus. (26) In response to P2X7R activation, caspase-1 cleaves the carboxylterminal from gasdermin-D (GSDMD), producing an active N-terminal fragment. (26–28) Its migration to the plasma membrane facilitates pore formation, cell expansion through potassium efflux, and rupture, which evokes the release of IL-1 β . (29,30) This promotes a vicious cycle whereby pyroptosis elicits the persistent activation of the P2X7R, while P2X7R activation continues to invoke pyroptosis. (19) Under these circumstances, immunomodulation of the P2X7R may represent an essential therapeutic avenue in the maintenance of inflammation associated with CAD.

Several classes of P2X7R antagonists have undergone clinical development in the last two decades. Although, human clinical trials have been mainly focused on autoimmune conditions such as Rheumatoid Arthritis and Crohn's Disease, where current therapeutic management is dependent on potent immunosuppressants such as methotrexate (23,24). However, P2X7R antagonists failed to achieve a therapeutic efficacy in these cohorts. Despite this, the P2X7R antagonist JNJ-54175446 demonstrated modest benefits in a cohort with major depressive disorder. (31) This cohort showed an additional benefit on systemic inflammation measured as a reduction in IL-1 β release from stimulated peripheral blood mononuclear cells (PBMCs). (31) This study supports the benefits of P2X7R antagonism outside of non-classical immunological disorders and may suggest the repurposing of these compounds for CAD.

While the innate and adaptive immune system are known to play a critical role in the atherogenic process, (6,32,33) no current pharmacotherapeutics are available to specifically target inflammation. While in the previous chapter, plaque stabilisation in a murine model was improved with the P2X7R antagonist PKT100, the short half-life has limited its clinical utility. (34) In an effort to improve the potency and metabolic stability of the first-generation P2X7R antagonist, a series of cyanoguanidine hybrids were developed that incorporated the adamantane moiety from PKT100. This also led to the identification of AZD9056 as a potent P2X7R scaffold in patients with CAD. As a second approach, bioisosteric substitution of the adamantane cage of AZD9056 resulted in the creation of the adamantane amide hybrids. This work aimed to characterise a novel series of P2X7R antagonists with improved physiochemical properties, designed to attenuate inflammation in CAD.

Methods

Compound Synthesis

The design and synthesis of the first-generation P2X7R antagonist, PKT100, was achieved by Dr Shane Michael Wilkinson and Professor Michael Kassiou from the School of Chemistry, University of Sydney (Australia). The compound was previously selected from a panel of 70 adamantane benzamide analogues due to its potency in pore-formation assays and improved metabolic stability compared to the initial adamantane benzamide lead compound. (35) The details of its design and its assessment in vitro are published elsewhere. (34) The second-generation P2X7R antagonists reported in this thesis were synthesised by Dr James O'Brien-Brown and Mr Jayson Gilchrist under the supervision of Professor Michael Kassiou from the School of Chemistry, University of Sydney (Australia). This series of compounds incorporated the cyanoguanidine linker previously reported by Abbott Laboratories (USA) and the adamantane moiety characteristic of PKT100. An initial panel of adamantly-cyanoguanidine hybrids was reported, which influenced further development of their analogues. (36)

A fourth-generation P2X7R antagonist was also developed at the School of Chemistry, University of Sydney (Australia) by Mr Jayson Gilchrist and Professor Michael Kassiou. The scaffold for these compounds was based on the adamantane amides reported by AstraZeneca (United Kingdom). (37) The purpose of this series was to improve the physiochemical properties and improved half-life of the previous compounds. Similar to the development of PKT100, a bioisosteric substitution strategy was employed to reduce the metabolic liability. Initial evaluation of the adamantanyl-cyanoguanidine and -amide scaffolds using Yo-Pro-1 dye (Thermo Fisher Scientific) uptake assay was performed by Mr Andre McKenzie under the supervision of Dr Eryn Werry and Professor Michael Kassiou at the School of Science, University of Sydney. This work is included in the thesis to demonstrate the workflow used in selecting the lead candidates. Compounds with a half-maximal inhibitory concentration (IC_{50}) value of < 80 nM were selected for further in vitro and ex vivo pharmacological characterisation. Compounds were evaluated in conjunction with commercially available P2X7R antagonists. They were selected based on their established potency in vitro (37–40) These compounds were previously explored in clinical trials and served as a reference for the ex vivo assays. All chosen compounds were determined to have an ideal pharmacokinetic and safety profile based on first-in-man clinical studies.

Benchmarking compounds AZD9056, GSK-1482160, JNJ-5175446, and JNJ-55308942 were purchased from MedChemExpress (USA).

The selective P2X7R agonist 3'-O-(4-benzoyl) benzoyl adenosine-5'-triphosphate (BzATP) was selected to activate the P2X7R. It is described as a synthetic ATP analogue with increased potency at the P2X7R. (41) BzATP was obtained from MedChemExpress and diluted it at a concentration of 5 mM in UltraPura DNase RNase-Free distilled water (Thermo Fisher Scientific, USA) on arrival. 90 µL aliquots of BzATP were stored at -80 for the study and thawed 10 minutes before use. For in vitro and ex vivo experiments, all compounds were reconstituted at 10 mM in pure dimethylsulfoxide (DMSO, Sigma-Aldrich, USA) and stored at -80°C for the duration of the experiments. Compounds were thawed 30 minutes before experiments, and any remainder of the aliquot was discarded after the experiment.

Cell culture

The human monocytic (THP-1) cell line was acquired from the American Type Culture Collection (ATCC) and was grown in suspension in RPMI-1640 (ATCC modified medium, supplemented with low sodium bicarbonate, L-glutamine, sodium pyruvate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, phenol red, and high glucose) (Gibco, USA). The medium was supplemented with 10% fetal bovine serum (FBS, AU origin, Gibco) in a T175 cell culture flask (Corning, USA).

In vitro Pore-formation

Mr Andre McKenzie completed initial in vitro drug screening at the University of Sydney under the supervision of Dr Eryn Werry and Professor Michael Kassiou. Pore formation in response to agonism is arguably one of the most common features of P2X7R. In response to P2X7R activation, a macropore forms, allowing non-selective molecules up to 900 Da to enter the cell. (42) The pore formation elicits NLRP3 assembly and the downstream maturation and release of interleukin-1 beta (IL-1 β) and interleukin-18 (IL-18) cytokines. (43) This assay is the backbone of P2X7R drug discovery and provides a robust and relatively cheaper option to screen compounds in a high-throughput manner. THP-1 cells, which harbours the endogenous P2X7R were prepared in the 96-well black side, clear bottom CellBIND plates (Corning, USA) at 1.5×10^5 cells and incubated overnight in reduced serum media (equivalent to 5% FBS) treated with 10 ng/mL interferon-gamma (IFN- γ , R&D Systems, USA) and 25 ng/mL lipopolysaccharide (LPS, Sigma-Aldrich) overnight at 37°C. Supernatant was removed from THP-1 cells, and 150 µL Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific) was added to wash cells. HBSS was then aspirated, and cells were treated with either (i) 0.001% DMSO in unstimulated basal wells, 1 mM BzATP diluted in 0.001% DMSO to induce pore formation, or (iii) 0.001 – 1 µM P2X7R antagonists in combination with 1 mM BzATP. In all wells, 1 µM of Yo-Pro-1 dye (Thermo Fisher Scientific) in HBSS. The time course was captured on a FLUOstar Omega Microplate Reader (BMG LABTECH, Germany) in the fluorescent detection mode at 491 nm excitations and 509 nm emission spectra. This indicated the binding of Yo-Pro-1 dye (Thermo Fisher Scientific) to cleaved nuclei acids and is an early indicator of apoptosis.(44)

In vitro IL-1 β and IL-18 release

The effectiveness of the cyanoguanidine and adamantane amide series to inhibit P2X7R-dependent IL-1 β and IL-18 release was assessed by quantitative protein detection in an enzyme-linked immunosorbent assay (ELISA). Cells were prepared by centrifugation and resuspension in a fresh medium without FBS supplementation. Cells were counted with a haemocytometer and resuspended at 2×10^5 cells per well. Cells were treated with 10 ng/mL IFN- γ and 25 ng/mL LPS to differentiate monocytes into macrophages and prime precursor cytokine production via the toll-like receptor 4. Cells were seeded into a 96-well, Corning CellBIND flat bottom transparent microplate (Corning) at a density of 2×10^5 cells per well at a volume of 100 μ L for 3 hours at 37°C in an enriched CO $_2$ environment. The supernatant was removed from wells, and cells were pre-treated with either (i) 0.001% DMSO if cells were to remain untreated or (ii) P2X7R antagonists resuspended in RPMI-1640 at a concentration between 0.001 - 1 μ M for 30 minutes at 37°C. After this time, pre-treatments were moved, and cells were gently washed with 150 μ L of phosphate-buffered saline (PBS) without magnesium and calcium (pH 7.4, osmolality 270-300 mOsm/kg, Gibco). PBS was then gently aspirated, and cells were treated with either (i) 0.001% DMSO in unstimulated basal wells, (ii) 1 mM BzATP diluted in 0.001% DMSO to stimulate cytokine release, or (iii) 0.001 – 1 μ M P2X7R antagonists in combination with 1 mM BzATP. Cells were incubated for 1 hour at 37°C with treatments. Supernatants were then collected and transferred to clean microcentrifuge tube (Eppendorf) and centrifuged for 5 minutes at 125 g at room temperature to remove cellular debris. The supernatant was then collected into a new microcentrifuge tube to eliminate potential contamination from pelleted cells. Samples were stored at -80°C until subsequent downstream analysis.

IL-1 β and IL-18 detection

Human IL-1 Beta/IL-1F2 and total IL-18 monoclonal DuoSet ELISA was purchased from R&D Systems. Capture antibodies were plated in 96-well clear, flat-bottom polystyrene microplates with high-bind treatment overnight at room temperature (R&D Systems). Before sample preparation, plates were washed with 0.05% Tween 20 in PBS, pH 7.2-7.4 diluted in Milli-Q water three times, with the solution removed between each wash. To prevent the non-specific binding of protein to the microplate, wells were then blocked with 0.1% BSA in PBS (R&D Systems) prepared in PBS (referred from here on as 'blocking buffer') for 2 hours at room temperature. Samples from in vitro experiments are thawed at room temperature and then placed on ice before preparation to avoid protein degradation. Samples were reconstituted 1:2 to detect IL-1 β and 1:1 to capture IL-18 in the prepared blocking buffer and incubated for 2 hours at room temperature. After these samples were aspirated, wells were washed with 0.05% Tween 20 in PBS, as detailed above. A second antibody aimed to detect the attached protein, commonly called the 'detection' antibody (R&D Systems), was then incubated in the wells for 2 hours at room temperature. After this time, the additional detection antibody was washed away as detailed above, and a 1:40 streptavidin (R&D Systems) conjugated with horseradish-peroxidase enzyme solution diluted in blocking buffer was included in each well for 20 minutes at room temperature. To remove unbound streptavidin (R&D Systems), the plate was again washed as detailed above, and a 1:1 solution of H $_2$ O $_2$ and tetramethylbenzidine (R&D Systems) was added to the well at room temperature for 20 minutes. This solution interacts with bound

horseradish-peroxidase enzyme to induce a colourimetric conversion on a spectrum from clear to dark blue. The colour change depends on the amount of IL-1 β , or IL-18 detected in the well. The reaction is stopped with 2N H₂SO₄, (R&D Systems) and the amount of specific protein in the sample was detected on a microplate reader (Varioskan LUX, Thermo Fisher Scientific) by measuring the amount of light absorbed by the reagent at a 450 nm wavelength with a 540 nm wavelength correction.

Subject cohort

Participants in **Cohorts one, two and three** were recruited through the BioHEART clinical study, which was approved by the Northern Sydney Local Health District Human Research Ethics Committee (ACTRN12618001322224). **Cohort one** was recruited to evaluate the efficacy of the cyanoguanidine-adamantane hybrids. In total, 34 participants with CT-defined CAD (CACS > 0 Agatston units (AU)) were compared to 21 participants without disease (CACS = 0 AU) and 24 participants with confirmed ST-elevated myocardial infarction (STEMI). Due to the nature of the study, acute phase protein measures such as C-reactive protein (CRP) were reported for STEMI participants, together with troponin concentration, but not for the CAD Cohort. **Supplementary Table 1** summarises the clinical and demographic characteristics of the cohort.

Based on the previous in vitro validation, only the most potent compounds from earlier studies were selected for benchmarking against the newer series. This approach provided a reference for the development of novel series and established performance thresholds to be reached for development. In an initial cohort, the magnitude of IL-1 β and IL-18 reduction with AZD9056 was compared to the first-generation P2X7R antagonist PKT100 and the second-generation compound PKT206. Compound prioritisation was applied based on their performance under high stimulation conditions, allowing for the determination of those with the greatest benefit against cytokine release. In **Cohort 2**, participants were recruited to evaluate potential structures that could serve as scaffolds for novel P2X7R antagonists. In total, 15 participants were determined to have CT-defined CAD and were compared to 12 participants without disease. **Supplementary Table 2** summarises the clinical and demographic characteristics of the cohort.

In **Cohort 3**, participants were recruited to evaluate the efficacy of the adamantane amide analogues in inhibiting IL-1 β and IL-18 release. Eight participants were determined to have CAD, whereas seven had no CAD. Three participants were recruited with STEMI. **Supplementary Table 3** summarises the clinical and demographic characteristics of the cohort. For both cohorts, a thorough history was obtained from each participant. In groups allocated to either CT-defined CAD or no CAD groups, a trained physician performed a CACS assessment used to stratify patients. CACS was analysed according to the standard anatomical arterial regions defined by the 17-segment model outlined by the Society of Cardiology Cardiovascular Computed Tomography. (45) Comparatively, participants in the STEMI cohort were recruited after percutaneous coronary intervention PCI for STEMI through Royal North Shore Hospital (Australia).

PBMC Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from participants recruited in this study. Previous evidence has shown that participants with CAD demonstrated heightened inflammation (12,46), making them excellent tools in drug discovery within an appropriate biological and genetic framework. Whole blood was collected by venous cannulation into vacuette lithium heparin tubes (Greiner Vacuette, Austria). Whole blood was diluted 1:1 with HBSS with calcium and magnesium (Thermo Fisher Scientific) and gently inverted three times. Diluted whole blood was layered on top of 13 mL of Ficoll-Paque media (density 1.077 g/mL, Cytiva, USA) in a 50 mL falcon tube and centrifuged at 800 $\times g$ for 15 minutes at room temperature with the break-off. Samples removed from the centrifuge were separated based on density, with the PBMCs forming the interphase between the plasma and the Ficoll mixed granulocytic region. Polynucleated immune populations such as neutrophils are removed in this step. PBMCs were collected into a 15 mL centrifuge tube (Corning) from the interphase region and centrifuged at 800 $\times g$ for 10 minutes at room temperature with the break on low. PBMCs were resuspended by gently swiping the side of the tube and resuspending in 10 mL of HBSS calcium and magnesium. The sample was inverted gently three times, and 10 μ L of resuspended PBMCs were removed and diluted 1:1 with 0.4% trypan blue for counting (Thermo Fisher Scientific). A valid cell count was determined by averaging the total cells counted manually using a haemocytometer and the total cells counted using the Countess 3 automated cell counter (Thermo Fisher Scientific). The centrifugation process was completed a second time at 800 $\times g$ for 10 minutes at room temperature with the break on low. The wash buffer was removed, and PBMCs were resuspended at 10×10^6 cells/mL in RPMI-1640 with 2 mM L-glutamine but without serum (Thermo Fisher Scientific) for downstream stimulation experiments.

Ex vivo patient-derived monocyte assay

After resuspension at 10×10^6 cells/mL in RPMI-1640 with L-glutamine (Thermo Fisher Scientific) but without serum and plated in duplicate in a 96-well flat bottom CellBIND microplate (Corning) at a density of 1.6×10^5 cells per well at a volume of 100 μ L. It is important to note that, compared to other methods used, PBMCs are not stimulated with LPS or IFN- γ . Instead, the proportion of monocytes that exhibit adhesion properties is likely reflective of a more mature monocytic population. Monocyte enrichment was achieved through plastic adhesion through incubation at 37°C for 3 hours in RPMI-1640 media without serum. Based on the in vitro potency of compounds, a single concentration of 200 nM was selected based on the median IC₅₀ across the series. Wells were either treated with (i) 0.001% DMSO in RPMI-1640 media or (ii) 200 nM of each compound reconstituted in RPMI-1640 media. Cells were washed gently with 150 μ L PBS without calcium and magnesium (Thermo Fisher Scientific) to remove any potential interference from RPMI-1640 media or non-adherent populations. PBS was then gently aspirated, and cells were treated with either (i) 0.001% DMSO in unstimulated basal wells reconstituted in PBS, (ii) 1 mM BzATP diluted in 0.001% DMSO to stimulate cytokine release through the P2X7R, or (iii) 200 nM P2X7R antagonists in combination with 1 mM BzATP in PBS for 30 minutes at 37°C. Supernatants were then collected and transferred to clean microcentrifuge tube and centrifuged for 5 minutes at 125 g at room temperature to remove cellular debris. The supernatant was then collected into a new microcentrifuge

tube to eliminate potential contamination from pelleted cells. Samples were stored at -80°C until subsequent downstream analysis.

In vitro potency analysis

In vitro assays were analysed in Microsoft Excel (USA) and GraphPad Prism software 10.0 (GraphPad Software Inc, USA). For pre-processing normalisation, absorbance values were exported raw into CSV format. Well-to-well variation was compensated by removing the 450 nm value from the 540 nm wavelength. The background signal from each well was corrected by the following equation: standard or unknown wells – blank well. Normalised data was then copied into GraphPad Prism, with the absorbance values for the standard equated with the corresponding predetermined concentration in an XY table. Unknown values were copied below for interpolation. To achieve a normal distribution, values were log-transformed (base 10) and then interpolated as a nonlinear regression, where the bottom was constrained to 0. For prospective analysis, values were untransformed with the equation $X=10^X$. Interpolated concentrations were processed to reflect the concentrations reached by adjusting for the dilution factor of the sample in the well. Values were plotted based on the percentage reduction compared to the BzATP-induced IL-1 β or IL-18 well for each treatment group. The basal measurement was removed to adjust for baseline secretion. These derived values were then subjected to statistical evaluation in GraphPad Prism software, where the percentage reduction was plotted compared to the dose-dependent logarithmic concentration. A nonlinear regression analysis was performed with a four-parameter variable slope to obtain the IC₅₀ value.

In vitro microsomal stability

The in vitro microsomal stability assays were completed as part of contracted work with the Centre for Drug Optimisation. The compounds stability was assessed through brief incubation with liver microsomes (Xenotech, USA) at 37°C. Compounds resuspended at 0.5 μ M were incubated against liver microsomes from humans, rats, and mice. The co-factor nicotinamide adenine dinucleotide phosphate (NADPH) was incubated with the test compound to initiate the reaction. After 60-minutes, acetonitrile was included with an internal standard to terminate the experiment where the supernatant was collected to be analysed on Liquid Chromatography-Mass Spectrometry Quadrupole system (Agilent, USA). The degradation of each of the compounds was measured and monitored throughout the 60 minutes. A control sample without NADPH was included to evaluate the potential contribution of NADPH-independent metabolic pathways. Samples were centrifuged at 9000 xg for 20 minutes at 4°C to remove any potential contamination before analysis. The in vitro intrinsic clearance was calculated from the first-order degradation constant, while the half-life was measured by the rate constant vs incubation time.

ProQuantum detection of cytokines from supernatants

For **Cohort 3**, supernatants from ex vivo monocyte assay was thawed at room temperature and immediately placed on ice after thawing. To achieve concentrations detected within the standard curve, samples were diluted

1:1 in assay dilution buffer. The protein standard was also diluted in assay dilution buffer to the recommended volume and left to stand at room temperature for 15 minutes. The protein standard, which is provided in the kit is composed of 500 ng of the recombinant protein being measured. A cocktail that contains antibody conjugates A and B in conjugate dilution buffer was prepared to bind analytes. Samples were prepared in a 1:1 ratio with the analyte binding solution in duplicates, sealed, vortexed briefly, and centrifuged at 3000 $\times g$ for 1 minute at room temperature. The plate was incubated at room temperature for 1 hour to achieve antibody-protein adhesion. After this time, a master mix containing the DNA ligase and thermostable DNA polymerase was prepared. The master mix was pipetted in multichannel format into each well, after which the plate was sealed with an opti-seal for PCR (Thermo Fisher Scientific), vortexed briefly and spun at 3000 $\times g$ for 1 minute at room temperature. The plate was read in a 384-well format on a 12K QuantStudio Flex Real-Time PCR System Thermo Fisher Scientific). Experiment parameters were set to standard curve or quantitation using TaqMan reagents. The reporter dye was set to FAM, with the quencher reported as NFQ-MGB and the passive reference as ROX.

ProQuantum Analysis

Data was exported as a CSV file. Duplicates from the run were averaged. Any CT values further than 1 CT away from each other were assessed for the true CT score based on other replicates. For interpolating unknown values in a standard curve, values were copied into Prism 10.0 (GraphPad Software Inc) for further analysis. Values were log-transformed (base 10) to achieve normal distribution. A variable slope with four parameters was established to interpolate the log-transformed (base 10) variables into the standard curve. No adjustments were made to the bottom and top values. The concentrations were achieved in picograms per mL by reversing the transformation of these values. The formula reported above in the section was used to derive percentage reduction in IL-1 β and IL-18 release.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 10 software (GraphPad Software Inc). For in vitro assays, percentage reduction values were plotted compared to the logarithmic concentration and fitted on a nonlinear regression. For patient cytokine secretion, values were analysed using a two-way ANOVA. To acquire means and standard deviation values for percentage reduction comparison between compounds, a bootstrap analysis method was applied to generate many simulated samples to obtain the most accurate means for the patient groups. For comparison of raw cytokine concentration, values were plotted after interpolating values from a standard. Adjustments for demographic and clinical variables used multiple regression analysis to acquire new value estimates based on the covariates. Values, which are presented as mean $\pm$ SEM. Significant was defined as $p < 0.05$.

Results

The cyanoguanidine-adamantane hybrids demonstrate potent inhibition of pore formation in THP-1 cells

Previous assessments of first-generation adamantanyl benzamide P2X7R antagonists showed bioisosteric fluorine-for-hydrogen substitution to the adamantane cage improved liver microsomal stability 2.3 times compared to the initial lead compound (0.30 minutes vs 0.13 minutes). (34) This resulted in the clinical development of PKT100 to treat residual inflammation in CVD. To enhance the potency and pharmacokinetic properties of PKT100, the cyanoguanidine motif attributed to A-804598 developed by Abbott Pharmaceutical was incorporated, due to its increased potency and substituted with an adamantane head group. Previous studies also showed that changes in the linker length between the adamantane and aryl portions improved the potency of cyanoguanidine hybrids. (36) Based on this evidence, changes in the activity of the P2X7R associated with modifications to the indazole moiety were explored.

An important function of the P2X7R is its ability to form non-selective macropores in response to ATP-induced stimulation. (41) These pores are associated with a loss of homeostasis and P2X7R-mediated pyroptosis. (27) The reduction in pore formation elicited by the cyanoguanidine P2X7R antagonists was evaluated through a logarithmic concentration-response curve. From the series, three compounds were demonstrated to achieve greater potency at the P2X7R compared to PKT100. PKT202 demonstrated the greatest benefits against pore formation from the series (18.6 ± 0.5 nM, **Table 1**). Comparatively, replacing an electronegative oxygen atom in exchange for nitrogen on the indazole moiety did not largely modify the potency of PKT201 (22.09 ± 1.19 nM, **Table 1**). In the case of PKT203, substituting the methyl group from position one to two on the pyrazole ring did not result in an observable difference in potency (22.2 ± 6.0 nM, **Table 1**). However, the transfer of the amino group from the fourth to the sixth position on the pyrazole ring reduced the potency of PKT204 2.3-fold compared to PKT203 (IC_{50} 51.0 ± 15.7 nM vs 22.2 ± 6.0 nM, **Table 1**).

Comparatively, the substitution of a pyrazole for an imidazole, producing a methylbenzimidazole moiety for compound PKT206, resulted in a small improvement in potency compared to the methylindazole equivalent PKT204 (IC_{50} 42.1 ± 12.5 vs 51.0 ± 15.7 nM, **Table 1**). The addition of a trifluoride on the benzamide, together with the substitution of a sulfur group in the benzimidazole ring and removal of the methyl group resulted in a 3.2-fold lower potency in pore formation with PKT207 compared to PKT202 (IC_{50} 59.8 ± 2.9 vs 18.6 ± 0.5 nM, **Table 1**). These results provide a rationale for evaluating the cyanoguanidine-adamantane hybrids in a more relevant in vitro assay. From these experiments, PKT201 and PKT202 were marked as potential leads in the series.

The cyanoguanidine-adamantane hybrids effectively inhibit IL-1 β release in THP-1 cells

Next, the effectiveness of the cyanoguanidine hybrids in reducing BzATP-induced IL-1 β release was explored. THP-1 cells were incubated with a range of logarithmic concentrations of P2X7R antagonists and 1 mM BzATP. This series was compared to the previously evaluated first-generation antagonist PKT100 and commercially available P2X7R antagonists GSK-1482160 and JNJ-54175446, which have undergone clinical development for

non-cardiovascular indications. Like the pore formation assay, PKT202 demonstrated the greatest potency from the series with a maximal inhibitory value of 6.73 ± 4.30 nM (**Table 1**). Compared to PKT100, replacing the amide with a cyanoguanidine linker for PKT200 improved the potency by 14-fold (6.73 ± 4.30 nM vs 93.4 ± 4.73 nM) (**Table 1**). PKT203 showed greater potency in inhibiting IL-1 β release, exceeding the concentrations achieved for PKT201. While both these compounds demonstrated equivalent IC_{50} values in pore formation, PKT203 demonstrated a 3-fold greater potency in IL-1 β release compared to PKT201 (11 ± 5.54 nM vs 33.0 ± 6.64 nM, **Table 1**).

This was also the case for PKT206, the only methylbenzimidazole structure in the series. For example, while PKT206 showed moderate potencies in the dye uptake assay, the concentrations reached in the IL-1 β release assay were comparable to PKT202 and PKT203 (IC_{50} 12.3 ± 7.49 nM, **Table 1**). Under these circumstances, the potency of PKT206 made it a priority for further exploration. PKT206 exhibited a 2.7-fold increase in potency compared to PKT201, which showed higher potency in pore formation. This data may suggest the potential biological relevance of the methyl group in receptor-drug interaction.

The fluorinated benzothiazole P2X7R antagonist PKT207 showed the weakest potency among this series. Since the compound was synthesised with the fluorinated bridgehead on the adamantane, indicative of PKT100, a small reduction in potency was expected in exchange for a longer half-life. The increased half-life associated with the trifluoride adamantanyl structure was attributed to an increase electronegativity associated with the fluorine atoms, making enzymatic degradation more difficult. (34) These characteristics were indicative of the adamantane benzamide series, from which PKT100 was selected as a lead compound. (34) PKT207 was demonstrated to be 3.8-fold less potent against IL-1 β release than dye uptake. Compared to the most potent in the series, PKT207 was found to be 34-fold less potent at the P2X7R compared to PKT202 (IC_{50} 225.9 ± 0.18 nM vs 33.0 ± 0.30 nM, **Table 1**). This reduction in potency beyond values attributed to PKT100 may be partly due to replacing the second nitrogen with sulfur atoms and removing the methyl group from the thiazole ring, which substantially affects the physiochemical properties of the compound. Under these circumstances, PKT207 was found to be unsuitable for clinical development due to its poor efficacy.

Since PKT202 showed better potency than its benzoxazole counterpart, the pyrazole equivalent of PKT206, PKT204 was evaluated next. In this instance, substituting a 6-methylindazole for a 6-methylbenzimidazole resulted in a 2.3-fold reduction in potency associated with PKT204 (IC_{50} 26.8 ± 10.85 nM, **Table 1**). To establish whether compounds demonstrate adequate potency to meet in-vivo requirements, the potency of commercially available P2X7R antagonists that have undergone clinical evaluation were also evaluated. GSK-1482160 was selected as the minimum potency required to meet therapeutic concentrations in vivo, as its development was terminated due to its failure to attain adequate concentrations in first-in-human studies. (47) In comparison, JNJ-54175446 was chosen as a benchmark of optimal potency, as the compound was demonstrated to reach adequate concentration to inhibit IL-1 β release with an IC_{50} of 82 ng/mL. (40) Apart from PKT207, all the

cyanoguanidine hybrids outperformed GSK-1482160 (56.03 ± 0.31 nM, **Table 1**). As previously demonstrated, the potency of JNJ-54175446 exceeded the concentrations reached for all other compounds (4.54 ± 5.37 nM, **Table 1**). Nonetheless, PKT202, PKT203, and PKT206 were confirmed to be within the range of JNJ-54175446 and acceptable for clinical development. This data continues to support the development of the cyanoguanidine series for the treatment of CAD.

The cyanoguanidine-adamantane hybrids effectively inhibit IL-18 release in THP-1 cells

Among the cytokines known to play a critical role in atherosclerosis, IL-18 release from leukocytes is hypothesised to destabilise atherosclerotic lesions. (48) Recent evidence from the CANTOS trial demonstrated that while IL-1 β neutralisation reduced interleukin-6 (IL-6) and CRP plasma concentration, IL-18 remained unchanged in response to IL-1 β neutralisation by Canakinumab. (49) This may indicate a residual source of inflammatory risk through IL-18. Given the recognised contribution of the P2X7R in downstream maturation and release of IL-18, the effectiveness of the cyanoguanidine series in reducing IL-18 from THP-1 cells was characterised. PKT206 demonstrated the greatest benefit against IL-18 reduction from the cyanoguanidine-adamantane hybrids with an IC_{50} value of 9.56 ± 0.233 nM (**Table 1**). The potency of PKT206 against IL-18 was nearly identical to the potency attributed to IL-1 β inhibition, underscoring its potential to modulate several critical inflammatory pathways. Its indazole derivative, PKT204, also demonstrated an IC_{50} for IL-18 release that was equivalent to its potency against IL-1 β (21.81 ± 3.89 nM, **Table 3**).

The two other lead candidates, PKT202 and PKT203, also showed potent half-maximal inhibitory values, although these values exceeded the concentrations previously reported in the IL-1 β release assay. For instance, the reported IC_{50} for PKT202 for IL-1 β release was 3.1-fold higher than for IL-18 (IC_{50} 21.06 ± 4.09 , **Table 1**). This was replicated for PKT203, which showed a 3.8-fold greater potency for IL-1 β inhibition compared to IL-18 (IC_{50} 11.0 ± 5.54 vs $42. \pm 6.43$, **Table 1**). This data suggests that the mechanism modulating cytokine maturation and release may not be linked entirely between IL-1 β and IL-18. This final point is particularly apparent when GSK-1482160 is evaluated against IL-18 release. Specifically, GSK-1482160 was demonstrated to be moderately potent against IL-1 β reduction but was utilised as a benchmark for the minimum threshold requirement for potency based on its behaviour in clinical trials. Against IL-18 release, GSK-1482160 was demonstrated to be 5.1-fold more potent in reducing IL-18 release compared to IL-1 β release, with an IC_{50} value of 10.9 ± 5.02 (**Table 1**). In IL-18 release assay, the potency of PKT206 was matched by GSK-1482160. Thus, against IL-18 release, GSK-1482160 was not sufficient as a benchmark for the minimal potency threshold.

A comparison of the other structures reported here showed a substantial improvement in potency for PKT100 and PKT207. Particularly in the case of PKT100, a 2.7 greater potency for IL-18 reduction was demonstrated compared to the IC_{50} values reported for IL-1 β inhibition (IC_{50} 34.5 ± 4.34 nM, **Table 1**). The same relationship was demonstrated with PKT201, which showed improved potency against IL-18 compared to IL-1 β (IC_{50} 13.1 ± 8.41 nM, **Table 1**). Moreover, the effectiveness of **PKT207** in reducing IL-18 release was 1.9-fold more

potent than values reported for IL-1 β reduction (117.4 ± 8.34 nM, **Table 1**). Under these circumstances, this data broadly suggests that inhibiting IL-18 with a P2X7R antagonist may be more effective than reducing IL-1 β , although there are some exceptions to this observation. This finding also implies that IL-18 and IL-1 β expression could be differentially regulated, although both relying on P2X7R activation for maturation and release. Overall, several cyanoguanidine hybrids are shown to be effective inhibitors of IL-18 release, which supports their further evaluation.

The cyanoguanidine-adamantane series effectively inhibit IL-1 β release from monocytes in patients with CAD

Next, the effectiveness of the cyanoguanidine hybrid series in patient-derived monocytes under biologically relevant conditions was explored. The assessment of these compounds in an appropriate ex vivo model provides evidence for the potential shortcomings of the individual compounds against various genetic and demographic backgrounds. Patients recruited for the study with CT-defined CAD (CACS >0 AU, n= 34) were compared to those without the disease (CACS = 0 AU, n=21). In the same cohort, participants were also compared to those hospitalised for urgent percutaneous coronary intervention (n=24). Participants were only included with confirmed STEMI. A description of this cohort is included in **Supplementary Table 1**.

The same high-throughput conditions used in the above THP-1 cell assay was applied to patient-derived monocytes. However, a single concentration of 200 nM was selected based on the estimated median IC₅₀ concentration required for half of the compounds to achieve maximal inhibition of IL-1 β release. Given the relatively small cohort size compared to other human studies, a bootstrap statistical method was used to provide unbiased estimates of the distribution of IL-1 β reduction for each cohort. The primary aim was to evaluate whether P2X7R antagonists exhibit differences in efficacy based exclusively on potency. As a secondary aim, the effectiveness of P2X7R antagonists was assessed to determine whether it was influenced by disease state or demographics.

Previous studies with P2X7R antagonists in human populations have shown that a compound must elicit a 90% reduction in IL-1 β in monocytes to achieve clinical efficacy. (38) Human trials with GSK-1482160 were terminated due to the inability of the compound to reach this criterion within safety margins. (38) These results were evaluated in an ex vivo patient-derived monocyte assay and showed that GSK-1482160 failed to exhibit a consistent therapeutic benefit across groups (**No CAD**, $2.43 \pm 27.10\%$, **CAD**, $28.3 \pm 10.3\%$, and **STEMI**, $41.0 \pm 10.8\%$, **Figure 1A-B**). Indeed, in some independent incidences, GSK-1482160 was observed to increase in IL-1 β release from monocytes. This largely correspond with patients with No CAD, who exhibited a 21-fold reduction in efficacy associated with IL-1 β inhibition compared to the STEMI group (**Figure 1A**). This may be related to its characterisation as an allosteric modulator of the P2X7R, which has been suggested to present certain limitations associated with the occupancy of the binding pocket. (19,38,50)

PKT207 showed the poorest efficacy of all compounds in inhibiting IL-1 β secretion from monocytes (**No CAD**, -25.0 $\pm$ 15.2%, **CAD**, -5.24 $\pm$ 9.82%, and **STEMI**, 8.17 $\pm$ 6.73%, **Figure 2A-B**). A close in vitro assessment determined the trifluorinated placement on the adamantanyl bridgehead substantially affected the potency of the compound at the P2X7R. Overall, the inclusion of fluorine atoms is likely to have influenced compound affinity for the receptor, given the increase in negative electron properties associated with the adamantane moiety. PKT100 contains the same strong electronegative fluorine atoms at the adamantane cage as PKT207 but comparatively demonstrates adequate inhibition against IL-1 β in STEMI and CAD participants (**No CAD**, 48.7 $\pm$ 15.4%, **CAD**, 63.8 $\pm$ 10.7%, and **STEMI**, 82.0 $\pm$ 4.74%, **Figure 1A-B**). However, the inclusion of the methoxy group provides some electron-donating effects, which may neutralise the overall electron-withdrawing effects on PKT100.

Based on this analysis, PKT206 achieved maximal inhibition of BzATP-induced IL-1 β release across cohorts (**No CAD**, 101 $\pm$ 5.3%, **CAD**, 97.4 $\pm$ 4.4%, and **STEMI**, 93.4 $\pm$ 2.1%, **Figure 1A-B**). These values corresponded with JNJ-54175446 (**No CAD**, 99.5 $\pm$ 1.8%, **CAD**, 97.0 $\pm$ 3.3%, and **STEMI**, 90.0 $\pm$ 4.8%, **Figure 1A-B**). PKT202, which was determined to be a potent inhibitor of dye uptake and IL-1 β release, was comparable in efficacy to PKT206 in STEMI and CAD participants (**STEMI**, 90.3 $\pm$ 3.64%, **CAD**, 84.2 $\pm$ 3.66%, **Figure 1A-B**). Despite variability in vitro experiments, PKT203 was also shown to have potent benefits in STEMI and CAD groups (**STEMI**, 79.9 $\pm$ 4.49%, **CAD**, 66 $\pm$ 11.6, **Figure 1A-B**). Although, subject to thresholds set for therapeutic effectiveness, PKT203 was unlikely to proceed for clinical development. Comparatively, PKT204 demonstrated potent inhibition of BzATP-induced IL-1 β in STEMI participants, with moderate efficacy in the CAD group (**STEMI** 87 $\pm$ 5.73%, **CAD**, 65.9 $\pm$ 7.35%, **Figure 1A-B**). However, in participants without CAD, PKT204 was 2.2-fold less effective against IL-1 β release compared to the STEMI group (**STEMI**, 87 $\pm$ 5.73%, **no CAD**, 40.4 $\pm$ 20.9, $p=0.034$, **Figure 1D**). In compounds with heightened benefits against IL-1 β reduction, such as PKT202, PKT206, and JNJ-54175446, no significant difference was detected between groups (**Figure 1D-F**). A deeper analysis of the limited therapeutic benefit of PKT204 in participants with no CAD showed a modest correlation with age (R_2 , 0.231, $p=0.0274$, **Figure 1G**). After adjustment for age and sex, the mean reduction in IL-1 β release elicited by **PKT204** was heightened in the CAD group compared to those without CAD (mean 43.65, SE = 20.45, $t = 2.14$, $p = 0.0361$). This data suggests that disease pathogenesis might partially explain the inconsistencies in compound efficacy.

Despite its early promise, PKT201 elicited poor inhibition of IL-1 β release across groups, particularly among those without CAD. Comparatively, PKT201 demonstrated moderate efficacy in reducing IL-1 β release from STEMI and CAD groups (44.9 $\pm$ 7.2% vs 40.8 $\pm$ 12.0%, **Figure 1A-B**). Amongst the no CAD group, the release of IL-1 β with PKT201 was poor (9.93 $\pm$ 18.2%, **Figure 1A-B**). This data continues to support the development of PKT206 and PKT202 from the cyanoguanidine hybrids. In some lower-potency compounds, a clear benefit in cytokine inhibition persists in participants with STEMI, which supports the therapeutic benefit of P2X7R antagonism in the acute setting.

Z make them unsuitable for further development. The relatively short half-life and increased clearance will likely affect long-term bioavailability in patients, despite potent inhibition of IL-1 β in human monocytes.

AZD9056 demonstrates potent inhibition of IL-1 β release in patients with CAD

The previous strategy employed, which involved combining the adamantane moiety with the cyanoguanidine linker, resulted in potent compounds with poor metabolic stability. It was revealed that each region of this compound was liable to metabolic degradation. (51) For this reason, this motif was terminated as a potential synthesis approach for P2X7R antagonists. Throughout this thesis, it has been shown that ex vivo patient-derived monocytes could be a high-throughput drug screening tool. Under these circumstances, this model was used to select a new scaffold for the development of P2X7R antagonists. To preserve the adamantane structure, which has been shown to improve potency, the efficacy of a commercially available adamantane amide P2X7R antagonist AZD9056 developed by AstraZeneca was investigated. The effectiveness of the compound in a cohort of patients with CT-defined CAD (CACS > 0 AU) against patients without CAD (CACS = 0 AU) was assessed. The clinical and demographic characteristics of this cohort are reported in **Supplementary Table 2**.

To determine the effectiveness of the AZD9056 scaffold, the reduction in IL-1 β release in response to BzATP stimulation compared to PKT206 and the first-generation antagonist PKT100 was evaluated. All compounds were evaluated at 200 nM, so only a portion would elicit a therapeutic response based on potency. In patients with CAD, AZD9056 potently reduced IL-1 β release ($95.30 \pm 4.6\%$, **Figure 2A**) PKT100, also characterised by the adamantane cage, showed a 33% reduction in efficacy compared to AZD9056 (**Figure 2A**). While both compounds are already derivatives of the initial adamantanyl benzamide structures proposed by AstraZeneca, different strategies were employed to enhance metabolic stability and retain potency. (35) For instance, the adamantane bridgeheads were a priority for the development of PKT100, whereas AstraZeneca included a long alkyl tail off the benzamide moiety.

The inclusion of the hydroxypropylamino group off the benzene ring has been previously shown to increase metabolic stability. (37) Compared to the initial adamantane structure, (35) the addition of the side chain on AZD9056 was demonstrated to have a moderate effect on potency (**Figure 2A**). Comparatively, PKT100 showed increased metabolic stability with the addition of the fluorine-for-hydrogen substitution but at the small disadvantage of lower potency at the P2X7R against IL-1 β release (**Figure 2A**). This may be attributed to the strong electronegativity associated with the fluorine atoms on the adamantane, which may affect the affinity of PKT100 at the binding site. Meanwhile, AZD9056 showed equivalent efficacy to PKT206 ($95.30 \pm 4.59\%$ vs $97.40 \pm 4.33\%$, **Figure 2A**). This data demonstrates that the AZD9056 scaffold would be an effective synthesis strategy for the development of novel P2X7R antagonists.

Patients with CAD demonstrate heightened BzATP-induced IL-1 β release from monocytes than those without CAD. (46) In an independent cohort, the first aimed to confirm this result and assess the effectiveness of

AZD9056 in a cohort with CAD. At baseline, there was no notable difference in BzATP-induced IL-1 β release between groups (**No CAD** 1.79 $\pm$ 0.58 pg/mL, **CAD** 23.59 $\pm$ 15.93, $p=0.744$, **Figure 2B**). Under stimulatory conditions, IL-1 β release was heightened in patients with CAD, which confirms previous findings (**CAD** 271.40 $\pm$ 93.54 pg/mL, **No CAD** 92.02 $\pm$ 36.29 pg/mL, $p=0.0090$, **Figure 2B**). As expected, concomitant treatment of monocytes with BzATP in the presence of AZD9056 reduced IL-1 β release by 82.3% (271.40 $\pm$ 93.54 pg/mL vs 46.67 $\pm$ 26.4 pg/mL, **Figure 2B**). Univariate analysis between the percentage reduction in IL-1 β caused by AZD9056 and CAD burden revealed a significant correlation with the CACS percentile ($R^2=0.305$, $p=0.033$). CACS centile scores the relative atherosclerotic burden compared to the population when adjusted for age and sex. This may suggest that AZD9056 may be more efficacious in a population with increased susceptibility to atherosclerosis. This data supports the idea that patients with CAD demonstrate a heightened inflammatory burden, which may be P2X7R-dependent.

Next, the relationship between CACS and the reduction in AZD9056 was modelled in a multiple linear regression model, where adjustments were applied for common modifiable risk factors (hypercholesterolemia and significant smoking history). Since statins have also been shown to have an anti-inflammatory benefit, dependent on low-density lipoprotein reduction, statin use was also included. (52) CACS percentile retained a significant positive association with the percentage reduction in IL-1 β release in CAD patients ($p=0.004$) (**Figure 2C**). The inclusion of statin treatment as a variable revealed a significant negative association between statin use and the effectiveness of AZD9056 ($p=0.0059$). However, therapeutic thresholds for IL-1 β reduction were reached for most statin participants, and this relationship disappeared when evaluated in a univariate model. Adjustment for hypercholesterolemia, smoking history and statins disclosed a closer relationship between AZD9056 percentage IL-1 β reduction and CACS centile beyond what was reported in the linear regression ($R^2=0.699$) (**Figure 2D**). Under these circumstances, the data provides further evidence that participants with increased susceptibility to CAD might benefit from immunomodulation strategies. Moreover, these results indicate that the AZD9056 is an appropriate scaffold for developing a novel series of P2X7R antagonists with improved translational potential.

Bioisosteric substitution on the adamantane cage increases solubility and metabolic stability

In response to the potent immunomodulatory benefits of AZD9056 in CAD patient-derived monocytes, a new series of compounds were synthesised using the same bioisosteric substitution employed with PKT100. These newly synthesised compounds were first evaluated in an in vitro dye uptake assay with THP-1 cells. To establish the efficacy of this novel series compared to the initial scaffold, AZD9056 was evaluated simultaneously. AZD9056 showed the greatest potency (IC₅₀ 19.8 $\pm$ 11.5 nM, **Table 3, S3A**). While adding a single fluorine atom on the adamantane bridgehead of PKT403 did not affect potency, trifluorinated PKT401 demonstrated a 2.6-fold reduction in potency compared to the initial scaffold (**Table 3, S3A**). A difluoride adamantane compound was synthesised to understand whether potency was concentration-dependent on the number of fluorine atoms. The potency of PKT402 was better compared to PKT401 but was overall less potent than PKT403 (**Table**

3, S3). This data suggests that the strong electronegativity linked with the fluorine atoms might influence the affinity of the moiety at the P2X7R.

To validate the potency of these compounds against the functional release of cytokines through a P2X7R-dependent mechanism, the THP-1 cytokine secretion assay was used. Like above, THP-1 cells were stimulated with 1 mM of BzATP in the presence of logarithmic concentrations of P2X7R antagonists. Both cytokines measured were detected with ELISA and expressed as the proportional reduction in BzATP release for a given concentration. The lead compound AZD9056 demonstrated the greatest potency against cytokine reduction, with IC₅₀ values reported in the low nanomolar range for both IL-18 and IL-1 β reduction (**IL-18** 9.62 $\pm$ 4.76nM, **IL-1 β** 15.3 $\pm$ 6.95 **Table 3, S3A**). Comparatively, PKT402 performed the weakest against IL-1 β release, with a reported IC₅₀ 1.6-fold above its reported potency for pore formation (64.2 $\pm$ 6.13 nM, **Table 3, S3C**). In contrast, the potency of PKT402 in IL-18 inhibition experiments was equal to values reported for dye uptake but also fell within the same range as the inhibitory potency reported for PKT401 (36.92 $\pm$ 4.48, **Table 3, S3A**). Compared to AZD9056, PKT403 exhibited lower potency in inhibiting IL-1 β but higher potency for inhibiting BzATP-stimulated release of IL-18 (**IL-1 β** 37.8 $\pm$ 4.81, **IL-18** 16.39 $\pm$ 4.10 nM, **Table 3, S3D**). Overall, the IC₅₀ values reached for PKT403 were determined to be adequate to proceed with next phase clinical development.

A substantial challenge in compound development is the rapid metabolic degradation of compounds liable to cytochrome P450 degradation. In a previous study, the fluorine substitution on PKT100 was found to effectively protected the adamantane core from metabolic degradation while preserving the geometric arrangement. (34) Under these circumstances, the metabolic stability of the PKT400 series compounds was explored in liver microsomes from humans, rats, and mice. PKT401, which features the trifluorine adamantanyl amide structure, showed a 25% improvement in half-life in human liver microsomes compared to AZD9056. (**PKT401** 191.0 minutes vs **AZD9056** 144.0 minutes, **Table 4**). This matches previous findings of the adamantane benzamide P2X7R antagonists, which shows that increased metabolic stability is associated with bioisosteric replacement. (34) However, in rodent liver microsomes, PKT401 demonstrated a substantial reduction in half-life compared to AZD9056 (**Table 4**). For instance, a 2.4-fold reduction was detected in half-life in mice compared to humans with PKT401 (**Table 4**). Despite this, the half-life in mice liver microsomes is 7.1-fold greater with PKT401 compared with PKT100 (**PKT401** 78.1 minutes vs **PKT100** 11 minutes, **Table 4**). (34) This data suggests that PKT401 shows adequate liver microsomal half-life to warrant further evaluation.

While the monofluoride compound PKT403 demonstrated the greatest potency in THP-1 cell pore formation and cytokine release experiments, its half-life was 2.5-fold shorter than values reported for PKT401 (**PKT403** 77.0 minutes vs **PKT401** 191.0 minutes, **Table 4**). This supports previous findings which showed that the fluorine atoms protect the metabolic liable sites on the adamantane head. However, this relationship was reversed in rodent liver microsomes. PKT403 was observed to have a greater half-life amongst rodent liver microsomes compared to humans. (**Figure 4**). For instance, the half-life in mouse and rat liver microsomes increased by 1.7

and 1.6-fold compared to humans, respectively (**Table 4**). This data might support the development of PKT403 for preclinical evaluation, given that evidence from previous studies has shown that AZD9056 demonstrates limited affinity for the rodent P2X7R. Comparatively, based on the in vitro half-life, PKT401 would be better suited for human clinical trials, given that the half-life suits the requirements for oral administration route.

Bioisosteric substitution influences efficacy in monocytes derived from CAD participants

Screening compounds against monocytes from participants with CAD closely recapitulates the in vivo reduction in IL-1 β from human trials. (31,37,38) Under these circumstances, PBMCs from patients with CAD could accelerate candidate selection in drug discovery and identify compounds with limited therapeutic value. Specifically, compounds with poor affinity against single-nucleotide polymorphisms can be identified early in the lead optimisation phase. Given these points, the efficacy of the adamantane amide series in inhibiting IL-1 β and IL-18 release in patients with CT-defined CAD and STEMI was evaluated. The parameters of this experiment were established above. To compare to the previously assessed P2X7R antagonists, monocytes were also treated with a single concentration of 200 nM of each compound, irrespective of potency. Treatment occurred concomitantly with 1 mM of the P2X7R agonist BzATP.

Participants with STEMI demonstrate greater inflammatory burden as a response to myocardial hypoxia and necrosis. (53,54) P2X7R antagonism with PKT400 compounds equally reduced BzATP-induced IL-1 β release, irrespective of potency (**Figure 3A-B**). In this cohort, the mean percentage reduction in IL-1 β release was approximately 88.3%, regardless of drug potency. This data suggests an improved efficacy of PKT400 series P2X7R antagonists to reduce P2X7R-mediated IL-1 β and IL-18 release. Similar values were reported for those without CAD (**Figure 3A-B**). In participants with CAD, PKT401 demonstrated the poorest efficacy from the series despite improved potency in THP-1 cell experiments compared to PKT402 (**Figure 3A-B**). PKT403 performed similarly to AZD9056 and showed a consistent reduction in IL-1 β . In fact, PKT403 and AZD9056 showed identical inhibition in IL-1 β release across cohorts compared to PKT401 and PKT402 (**Figure 3A-B**). This data suggests the clinical translation of PKT403 for treating CAD.

The effectiveness of this series of P2X7R antagonists in reducing IL-18 release in response to BzATP stimulation was explored. Overall, P2X7R antagonism reduced the release of IL-18 across cohorts (**Figure 3C-D**). While most compounds were demonstrated to inhibit IL-1 β and IL-18 release equally, there were minor fluctuations in effectiveness. For instance, PKT401 demonstrated greater efficacy in reducing IL-18 than IL-1 β in the CAD group, whereas PKT402 and AZD9056 demonstrated a minor drop in effectiveness in reducing IL-18 compared to values reported for IL-1 β (**Figure 3C-D**). While statistical analysis indicates no significant differences between compounds in their effectiveness to inhibit IL-18 or IL-1 β release, it is expected that a P2X7R antagonist should elicit 90% inhibition against IL-1 β release to achieve efficacy. (38) Under this criterion, PKT403 holds the greatest promise for translation into preclinical studies based on its efficacy in reducing IL-1 β and IL-18 release.

Recent cardiovascular trials have moved further downstream of IL-1 β to IL-6 as a potential therapeutic target, given the interest in CRP as a measure of inflammation in CAD. (55–57) Unlike IL-1 cytokines, IL-6 is synthesised in its mature form and is not cleaved by caspase-1. (58,59) Alternatively, its synthesis is linked with the release of IL-1 β through activation of transcription factor NF- κ B. (58) Under these circumstances, IL-6 release from patient-derived monocytes was evaluated. Participants with arthritic conditions were excluded from the study, given the distinct role of inflammation secondary to cartilage degradation. The first aim was to establish whether monocytes from CAD and STEMI participants demonstrated heightened baseline concentrations of IL-6. There was no observable difference in IL-6 release under baseline conditions between no CAD, CAD, and STEMI groups (**No CAD** 1.08 $\pm$ 0.66 pg/mL, **CAD** 2.90 $\pm$ 1.24 pg/mL, and **STEMI** 3.63 $\pm$ 0.59, **Figure 3E**). Similar to the patterns observed in IL-1 β release after BzATP-stimulation, IL-6 was measured from monocytes after BzATP-dependent P2X7R activation. Under stimulatory conditions, there was no discernible difference in IL-6 release from participants in response to BzATP stimulation compared to baseline concentrations (**No CAD** 6.63 $\pm$ 1.92 pg/mL, **CAD** 10.22 $\pm$ 1.24 pg/mL, and **STEMI** 14.32 $\pm$ 5.5, **Figure 3E**). Under these experimental conditions, P2X7R antagonists were not shown to reduce IL-6 release. This data suggests that longer incubation times may be required to elicit IL-6 release. Despite this, the significant reductions in IL-1 β and IL-18 in patient-derived monocytes in response to P2X7R antagonism with PKT400 series compounds suggest an overall potent immunomodulatory effect of the fluorinated adamantane amide P2X7R antagonists in CAD.

Discussion

Herein, the development of two novel series of P2X7R antagonists for treating dysregulated inflammatory signalling associated with CVD are reported. The first series characterised incorporated a cyanoguanidine linker with the adamantane moiety, which has previously been shown to improve the metabolic stability of P2X7R antagonists. Overall, this series was more potent than the previous first-generation compound PKT100. In vitro evaluation identified PKT202 and PKT206 as lead candidates for clinical development. Using monocytes isolated from patients with CAD and STEMI, PKT206 was shown to be an effective inhibitor of BzATP-induced IL-1 β release. PKT206 was also demonstrated to perform equivalently to JNJ-54175446, which has recently undergone clinical evaluation in a cohort with major depressive disorder. (31) However, human and rodent liver microsomal analysis determined that these compounds had relatively short half-lives, making these compounds poor candidates for clinical development. Instead, a second series of compounds was characterised based on the AZD9056 scaffold. Bioisosteric substitution of the adamantane bridgeheads with fluorine atoms improved metabolic stability with the trifluorine adamantane PKT401. Evaluation of compound effectiveness to inhibit cytokine release in monocytes isolated from patients with CAD and STEMI determined that PKT403 and AZD9056 performed equivalently in reducing IL-18 and IL-1 β release. While IL-18 was potently inhibited in response to P2X7R antagonism, some minimal variability was shown with PKT401 against IL-1 β release. This data supports the clinical development of PKT403 as a novel P2X7R antagonist for the treatment of CAD. While the cyanoguanidine-adamantane compounds were deemed inappropriate for clinical development, the improved potency of PKT202 and PKT206, coupled with their consistent inhibition across cohorts may indicate a potential

opportunity for their development as positron emission imaging for vascular inflammation. Current diagnostic strategies in the detection of CAD are dependent on risk-based stratification.. (60) Atherosclerosis in its subclinical phase is unlikely to be identified with currently available technologies.

While the neutralisation of a single cytokine has demonstrated benefits against future adverse cardiovascular events in patients with CAD, a substantial residual inflammatory risk remains tied to the release of other pro-inflammatory cytokines. (8,49) This was evident in the CANTOS trial, which showed significant reductions in IL-6 without substantially benefiting IL-18 concentrations after IL-1 β neutralisation. (49) Comparatively, the P2X7R has been demonstrated to play a critical role in the modulation of IL-1 β and IL-18 maturation and release. (61) The crucial biological role of the P2X7R as a potent modulator of IL-18 and IL-1 β release was reconfirmed in this study. There were also many instances where P2X7R antagonists demonstrate variability in their ability to reduce both cytokines, which may indicate two distinct biological pathway which modulate IL-18 and IL-1 β release. Previous research have shown that the synthesis of precursor IL-1 β relies on of toll-like receptor activation of the NF- κ B pathway. (62) These receptors have been demonstrated to be deleterious in atherosclerosis, where their upregulation is associated with increased inflammatory burden. (63) The baseline expression of IL-1 β was shown to be negligible in healthy humans, whereas IL-18 is found to be continuously expressed in healthy human monocytes. (64) This response is suggested to be the result of type I interferon receptors, which have been shown to elicit substantial suppressive effects on IL-1 β expression through the interleukin-10-STAT3 pathway.(65) This tight regulation over IL-1 β likely reflects its deleterious activity in normal physiology. Furthermore, of type I interferon receptors were also demonstrated to mediate the NLRP3 inflammasome complex, and in turn can reduce P2X7R-dependent IL-1 β maturation. (65) Under these circumstances, these finding suggests that both cytokines should be evaluated against P2X7R antagonists before determination of a lead candidate.

The use of PBMCs isolated from patients with CT-defined CAD and STEMI provides an excellent model for evaluating the efficacy of compounds targeting cardiovascular inflammation. This model takes into consideration complex priming factors related to host environmental, disease-related, and genetic factors, and it has substantial advantages over commercial cell lines. Additionally, the inclusion of non-invasive imaging allows for better stratification of patient cohort based on at-risk markers. From the first series, PKT206 outperformed the series and was associated with potent reductions in IL-1 β across cohorts. PKT202 and PKT204 were also potent across cohorts, although efficacy was reduced in participants without CAD. GSK-1482160 was included as part of the series as the threshold requirement for progression. In phase-1 clinical trials, GSK-1482160 was terminated due to poor efficacy against IL-1 β inhibition, which reflected poor therapeutic potential in healthy participants within safety margins. The data presented here reflects this relatively poorer efficacy compared to other commercially available compounds such as AZD9056. Additionally, this also confirms the use of isolated patient-derived monocytes to stratify drug candidates. The second series of compounds showed similar benefits to the ex vivo assay for compound evaluation against IL-18 and IL-1 β . AZD9056 and PKT403

performed equivalently in patient PBMCs, while small reductions in efficacy were seen with the di- and trifluorinated compounds.

Although several compounds were determined to be relatively potent in vitro assays, their efficacy in patient-derived monocytes was variable and may be influenced by pharmacological factors beyond potency. For instance, several of the compounds screened, such as PKT201 and PKT203, demonstrated potent half-maximal inhibitory concentrations in assays but failed to elicit a substantial response in patient-derived monocytes. The pharmacological properties of the P2X7R are linked to the presence of approximately eleven non-synonymous single-nucleotide polymorphisms (SNPs). (66–69) Many of these have been demonstrated to induce structural modifications to the receptor, influencing its activity in response to ATP. (67) For example, a single-based pair mutation resulting in an amino acid change from glutamic acid to alanine at position 496 produced a protein with reduced capacity to form pores. (70) Comparatively, an alanine for threonine mutation at position 348, which maps a modification on the transmembrane domain, is associated with greater release of IL-1 β and ethidium uptake. (71) Based on whether a compound binds to allosteric or orthosteric pockets, these SNPs might also affect the efficacy of the compound at the receptor. Studies with PKT100, which is predicted to bind through allosteric means, demonstrated that an the from glutamic acid to alanine at position 496 mutation reduced potency 1.9-fold. (34) Several of these SNPs attributed to the P2X7R may occur together, creating further complexities around the selection of a candidate.

There was also a heightened benefit of several compounds in STEMI participants beyond what was demonstrated in other groups. PKT203 and PKT204 failed to reach the minimum clinical development threshold in the CAD group despite reaching those margins in those with STEMI. Despite substantial improvements in potency with the cyanoguanidine series, these benefits were similar to those demonstrated with PKT100 in the CAD population. This may reflect the potential relevance of this pathway in acute myocardial infarction. (53,72,73) In the previous chapter, P2X7R expression across classical monocytes, and CD1c+ myeloid dendritic cells was reduced in STEMI patients It was hypothesised whether the P2X7R could be shed or internalised as a protective mechanism against large-scale pyroptosis. In myocardial infarction, the death of cardiomyocytes releases damage-association molecular patterns, including ATP, (74) which provides a persistent source of material for P2X7R activation. Under these circumstances, targeting the P2X7R may represent an important therapeutic strategy against inflammation after acute STEMI.

Similar to first-generation P2X7R antagonist PKT100, the inclusion of the fluorine atoms on the adamantane cage increased the half-life in human liver microsomes. (34) This is a common synthesis strategy since fluorine is relatively small and unlikely to affect the overall size or shape of a compound. (75) Moreover, in both circumstances, a single fluorine atom on the benzamide reduced the half-life. (34) This may be the result of cytochrome P450, which has been demonstrated to target carbon to hydrogen bonds. (76) However, in the trifluoride structure, these metabolically labile sites are protected by strong polarised bonds formed by the

strongly electronegative fluorine atom and the carbon on the adamantane bridgehead. The half-life of these compounds was reversed in rodents, where mice and rats demonstrated greater metabolic stability with the single fluorine structure than the trifluoride adamantane hybrid. This is not an uncommon finding, as there are well-known differences in the cytochrome profile between humans and rodents. (77) However, a substantial question remains about the ideal properties required to achieve efficacy in vivo. The half-life attributed to PKT403 may be sufficient to achieve the daily concentrations required for biological inhibition. For instance, the P2X7R antagonists CE-224,535 developed by Pfizer showed a half-life of approximately 77 minutes in pharmacokinetic studies in monkeys. (78) Despite this, the trough concentrations reached in humans were 25 times greater than the estimated IC₉₀ value for inhibiting IL-1 β release in a whole blood stimulation assay. (79)

The data here also demonstrates that P2X7R-dependent IL-1 β from patient-derived monocytes is closely correlated with their CACS percentile, which supports the important pathophysiological role of this pathway but may also have potential translational significance. Current diagnostic tools for the detection of vascular inflammation have been limited to CRP, which has demonstrated poor utility at an individual level. In a cohort with CAD, a weak association was shown between CRP and CAD, where the most profound association was in obese participants. (80) However, there is a current unmet need for the discovery of blood-based biomarkers for the detection of subclinical atherosclerosis. This is particularly critical in a subpopulation of first-time myocardial infarction patients who present without traditional modifiable risk factors. (81,82,82,83) Identification of these novel biological markers has the potential for the discovery of untargeted pathways that may be key in addressing the missing underlying biology in atherosclerosis. (84) A primary concern with the use of CRP in vascular inflammation is its limited causal inference to atherosclerosis independently of IL-1 β and IL-6. (85) Comparatively, IL-1 β has been known to be causal in vascular inflammation for decades. Under these circumstances, fluctuations in its detection may provide a critical biomarker for those most susceptible to CAD in the subclinical phase. Moreover, its use as a potential companion diagnostic for the identification of participants that would most benefit from treatment with a P2X7R antagonist has the capabilities to accelerate cardiovascular human trials, which currently require extensive time and large cohorts to demonstrate efficacy above 25%.

Limitations

Although this study offers valuable insights into the development and characterisation of a novel series of P2X7R compounds to treat the inflammatory contribution of CAD, several limitations exist. The effectiveness of IL-1 β and IL-18 reduction in THP-1 concentration-response curves was evaluated but in patient-derived monocytes only IL-1 β was evaluated for the cyanoguanidine-adamantane series. IL-18 is associated with several cardiovascular disorders and would serve as an important marker to assess in the future. In addition, recent studies have pointed to IL-6 as a potential biomarker in atherosclerotic cardiovascular disease, with future cardiovascular outcome trials focused on this pathway in patients with chronic kidney disease. (86) The efficacy of this series was not evaluated against SNPs from patient-derived monocytes. In a future study, compound

efficacy in response to SNPs of the P2X7R will be evaluated. For the fluorinated adamantane-amide series compounds, the efficacy of IL-18 was evaluated across patients, which provided useful information on the effectiveness of these compounds in a human setting. However, the evaluation of IL-6 under these experimental parameters was insufficient to elicit IL-6 release under stimulated conditions. Under these circumstances, further optimisation of the assay is required to evaluate IL-6 release. It is hypothesised that longer incubation times are required to measure IL-6, given that its synthesis is partially dependent on IL-1 β .

Conclusions

In conclusion, these results showed that combining the cyanoguanidine hybrids demonstrated improved potency against IL-1 β and IL-18 release. PKT206 and PKT202 exhibited the greatest benefits across cohorts, with a decrease in physiological concentrations of IL-1 β comparable to JNJ-54175446. However, the fate of these series was sealed by their liability to degradation, making them inappropriate for the treatment of CAD. Under these circumstances, the synthesis of the adamantane amide structures resulted in the discovery of a series of compounds with improved potency and metabolic stability. Under these circumstances, this chapter supports the clinical development of PKT403 or AZD9056 for the treatment of CAD.

Future Directions

The immediate next step for this study is to evaluate the PKT400 series compounds in rodents to establish the *in vivo* pharmacokinetic properties. For utility in animal models, the concentrations required to reach therapeutic concentrations must be determined within safety margins. As the primary interest is in compounds aimed to stabilise atherosclerotic plaques, the most suitable compound will be evaluated in a model of tandem stenosis, which was reported back in chapter 4. Funding has also been acquired to investigate these compounds in pulmonary hypertension, where a substantial unmet need remains.

A substantial question also remains about whether these compounds will retain affinity at the rodent receptors. Previous studies have proposed that AZD9056 lacks affinity for rodent receptors. (87) This was alluded to in a preclinical streptococcal cell wall rat model, which utilised a rat-specific analogue of AZD9056. (87) Cloning of the mouse, rat, and human P2X7R has begun to evaluate the interspecies variability associated with adamantane-amide hybrids. In a previous study, the initial adamantane amide structure reported by AstraZeneca had limited affinity at the mouse receptor compared to the human. The inclusion of the fluorine-for-hydrogen substitution improved the potency against the mouse receptor. (34) This may inform the decision to use one P2X7R antagonist for preclinical studies, while developing the other for human clinical trials.

As part of this project, the aim is also determined whether these compounds bind as allosteric or orthosteric antagonists. Previous research indicates promiscuous inhibition may be favoured over allosteric antagonism. (19,88) An allosteric modulator was considered less effective as bound ATP elicits a conformational change to

the receptor and shrinks the allosteric pocket. (88) This will allow better characterisation of an ideal compound to proceed for clinical development.

In addition to the ex vivo monocyte experiment, which has demonstrated value as an indicator of patient efficacy in a physiologically relevant context, the top ten non-synonymous polymorphisms associated with the P2X7R will also be evaluated. In particular, this study will focus on whether BzATP-induced stimulation is affected by conformational changes to the P2X7R. The evaluate will also determine whether the detected SNPs are associated with the effectiveness of P2X7R antagonists in reducing IL-1 β release.

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Table 1. Potencies of the cyanoguanidine-adamantane series in THP-1 cells evaluated in Yo-Pro-1 dye uptake, IL-1 β and IL-18 release assays.

Compound	IC ₅₀ (dye-uptake, nM $\pm$ SEM) ^a	IC ₅₀ (IL-1 β release, nM $\pm$ SEM) ^b	IC ₅₀ (IL-18 release, nM $\pm$ SEM) ^c
PKT201	22.09 $\pm$ 1.19	33.0 $\pm$ 6.64	16.0 $\pm$ 8.41
PKT202	18.6 $\pm$ 0.5	6.73 $\pm$ 4.30	21.1 $\pm$ 4.09
PKT203	22.2 $\pm$ 6.0	11.0 $\pm$ 5.54	42.0 $\pm$ 6.43
PKT204	51.0 $\pm$ 15.7	26.8 $\pm$ 10.95	21.9 $\pm$ 3.89
PKT206	42.1 $\pm$ 12.5	12.3 $\pm$ 7.49	9.56 $\pm$ 2.33
PKT207	59.81 $\pm$ 2.91	225.9 $\pm$ 4.31	117.4 $\pm$ 8.34
PKT100		93.4 $\pm$ 4.73	34.5 $\pm$ 4.34
GSK-1482160		56.03 $\pm$ 5.82	10.8 $\pm$ 5.02
JNJ-54175446		4.54 $\pm$ 5.37	0.34 $\pm$ 5.10

^a IC₅₀ values for human P2X7R dye-uptake assay using THP-1 cells. Data presented as the mean of 5 experiments with the uncertainty reported as the SEM.

^b IC₅₀ values for human P2X7R IL-1 β release assay using THP-1 cells. Data presented as the mean of replicate experiments with the uncertainty reported as the SEM.

^c IC₅₀ values for human P2X7R IL-18 release assay using THP-1 cells. Data presented as the mean of replicate experiments with the uncertainty reported as the SEM.

^d IC₅₀ values for human P2X7R dye-uptake assay using THP-1 cells not assessed for PKT100, GSK-1482160 and JNJ-54175446.

Table 2. Metabolic stability assessment determined in liver microsomes from rodents and humans.

Compound	Species	T1/2 (min) ^a	Clint, in vitro (μL/min/ mg protein) ^b	cLogP ^c
PKT201	Human	8.1	170.2	3.2
	Rat	2.1	668	
	Mouse	2.9	710.2	
PKT202	Human	2.7	517.5	3.3
	Rat	<1.5	>920	
	Mouse	<1.5	>920	
PKT204	Human	6.9	201.9	3.3
	Rat	1.7	823.5	
	Mouse	2	682.9	
PKT207	Human	7.6	181.3	2.5
	Rat	6.4	218.3	
	Mouse	2.5	548.4	
PKT100	Human	66.8	20.7	2.4
	Rat	33.7	41.1	
	Mouse	11.0	124	
JNJ-55308942	Human	>490	<3.0	2.0
	Rat	>490	<3.0	
	Mouse	266	5.2	

^a Half-life (T1/2 (min)) determined by in vitro liver microsome studies on mouse, rat, and human microsomes.

^b In vitro intrinsic clearance Clint, in vitro (μL/min/mg protein)), calculated from the first order degradation rate constant (min⁻¹) from microsomal assay.

^c Lipophilic efficiency: LiPE = pIC₅₀ – cLogP.

^d Data reported as the mean of 3 experiments.

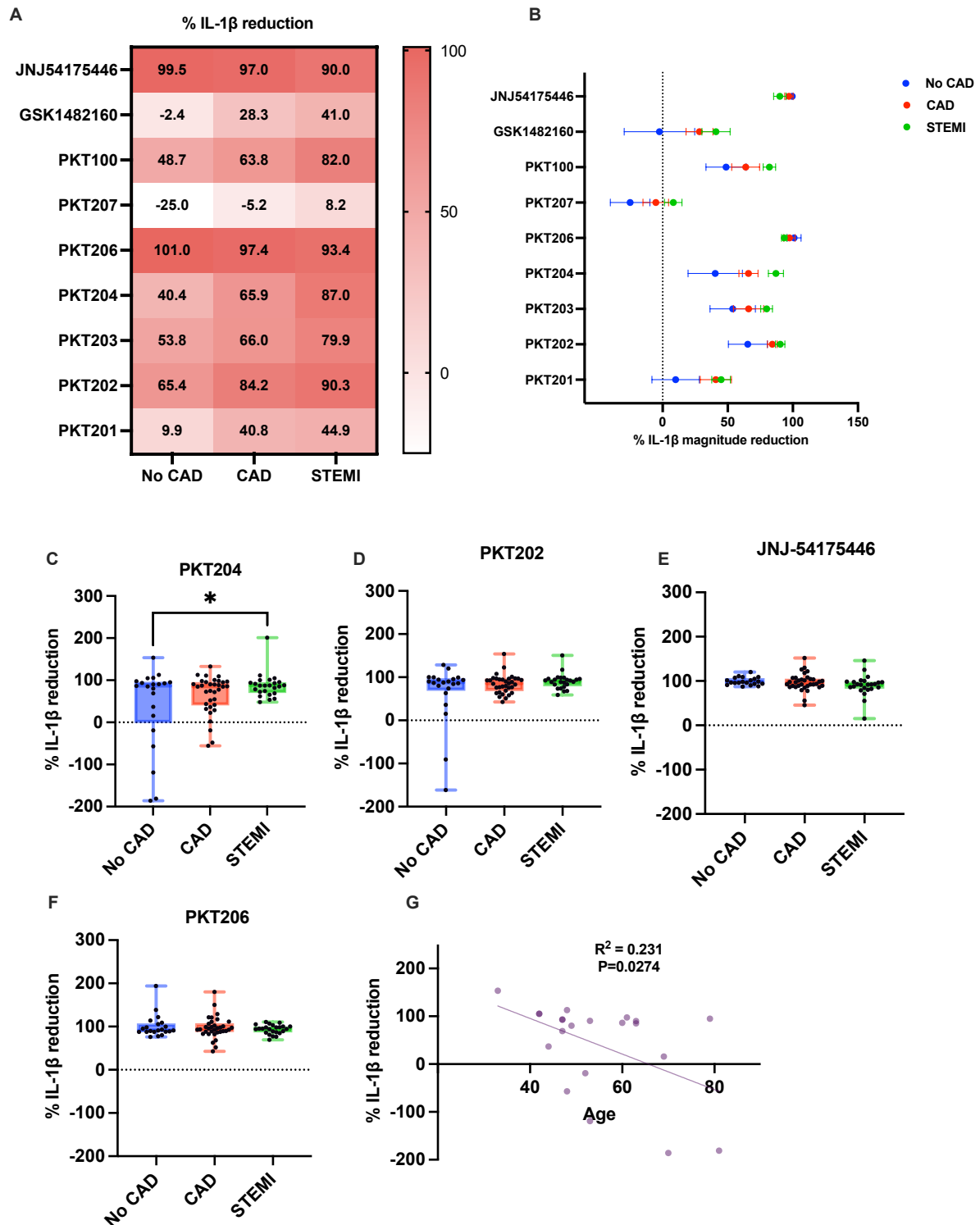


Figure 1. The efficacy of the cyanoguanidine adamantane hybrids against monocytes derived from patients with CAD. A) Heat map demonstrating the percentage reduction in IL-1 β elicited after BzATP stimulation in patients with no CAD, CAD, and STEMI. **B)** Visual representation of the percentage reduction in IL-1 β elicited in patients with no CAD, CAD, and STEMI. **C-F)** Bar graphs showing the percentage reduction in IL-1 β release amongst no CAD, CAD, and STEMI participants for PKT202, **D)** PKT204, **E)** JNJ-54175446, **F)** PKT206, and **G)** Linear regression model demonstrating the relationship between percentage IL-1 β release with PKT204 and age in participants with no CAD ($n = 21$). Values are expressed as mean $\pm$ SD. * $P < 0.05$

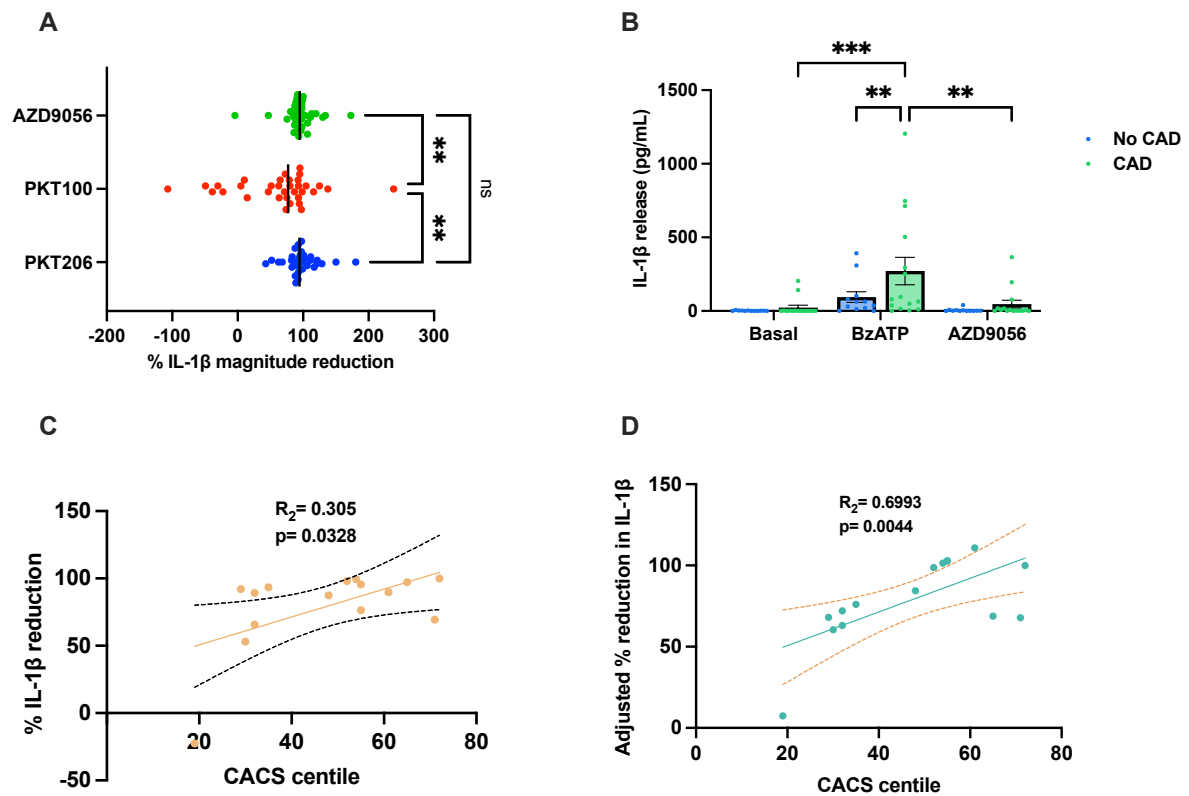


Figure 2. AZD9056 demonstrates a potent reduction of BzATP-induced IL-1 β release. **A)** Percentage magnitude reduction in IL-1 β in response to AZD9056, PKT206, and PKT100 inhibition in CAD monocytes. **B)** BzATP-induced IL-1 β release is inhibited by AZD9056 in patients with CAD. **C)** Linear regression between percentage reduction in IL-1 β release compared to CACS centile, **D)** Multiple linear regression adjusting for CACS centile, hypercholesterolemia, statin, and smoking history. Values are expressed as mean $\pm$ SEM. ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.0001$.

Table 3. Potencies of the fluorinated adamantane series in THP-1 cells evaluated in Yo-Pro-1 dye uptake, IL-1 β and IL-18 assays.

Compound	IC ₅₀ (dye-uptake, nM $\pm$ SEM) ^a	IC ₅₀ (IL-1 β release, nM $\pm$ SEM) ^b	IC ₅₀ (IL-18 release, nM $\pm$ SEM) ^c
AZD9056	19.8 $\pm$ 11.47	15.3 $\pm$ 6.95	9.62 $\pm$ 4.85
PKT401	51.27 $\pm$ 12.32	48.6 $\pm$ 5.31	33.48 $\pm$ 3.48
PKT402	39.70 $\pm$ 4.17	64.2 $\pm$ 6.13	36.92 $\pm$ 4.48
PKT403	22.0 $\pm$ 3.43	37.8 $\pm$ 4.81	16.39 $\pm$ 4.10

^a IC₅₀ values for human P2X7R dye-uptake assay using THP-1 cells. Data presented as the mean of 5 experiments with the uncertainty reported as the SEM.

^b IC₅₀ values for human P2X7R IL-1 β release assay using THP-1 cells. Data presented as the mean of replicate experiments with the uncertainty reported as the SEM.

^c IC₅₀ values for human P2X7R IL-18 release assay using THP-1 cells. Data presented as the mean of replicate experiments with the uncertainty reported as the SEM.

Table 4. Metabolic stability assessment determined in liver microsomes from rodents and humans.

Compound	Species	T1/2 (min)	Cl _{int} , in vitro (μ L/min/mg protein)
AZD9056	Human	144.0	9.6
	Rat	124.8	11.1
	Mouse	113.2	12.2
PKT401	Human	191	7.2
	Rat	84.8	16.4
	Mouse	78.1	17.7
PKT402	Human	92	15
	Rat	59	23.5
	Mouse	79.9	17.4
PKT403	Human	77.0	18.0
	Rat	127.0	10.9
	Mouse	133.0	10.5
PKT100	Human	66.8	20.7
	Rat	33.7	41.1
	Mouse	11.0	124

^a Half-life (T1/2 (min)) determined by in vitro liver microsome studies on mouse, rat, and human microsomes.

^b In vitro intrinsic clearance (Cl_{int}, in vitro (μ L/min/mg protein)), calculated from the first order degradation rate constant (min⁻¹) from microsomal assay.

^c Data reported as the mean of 3 experiments.

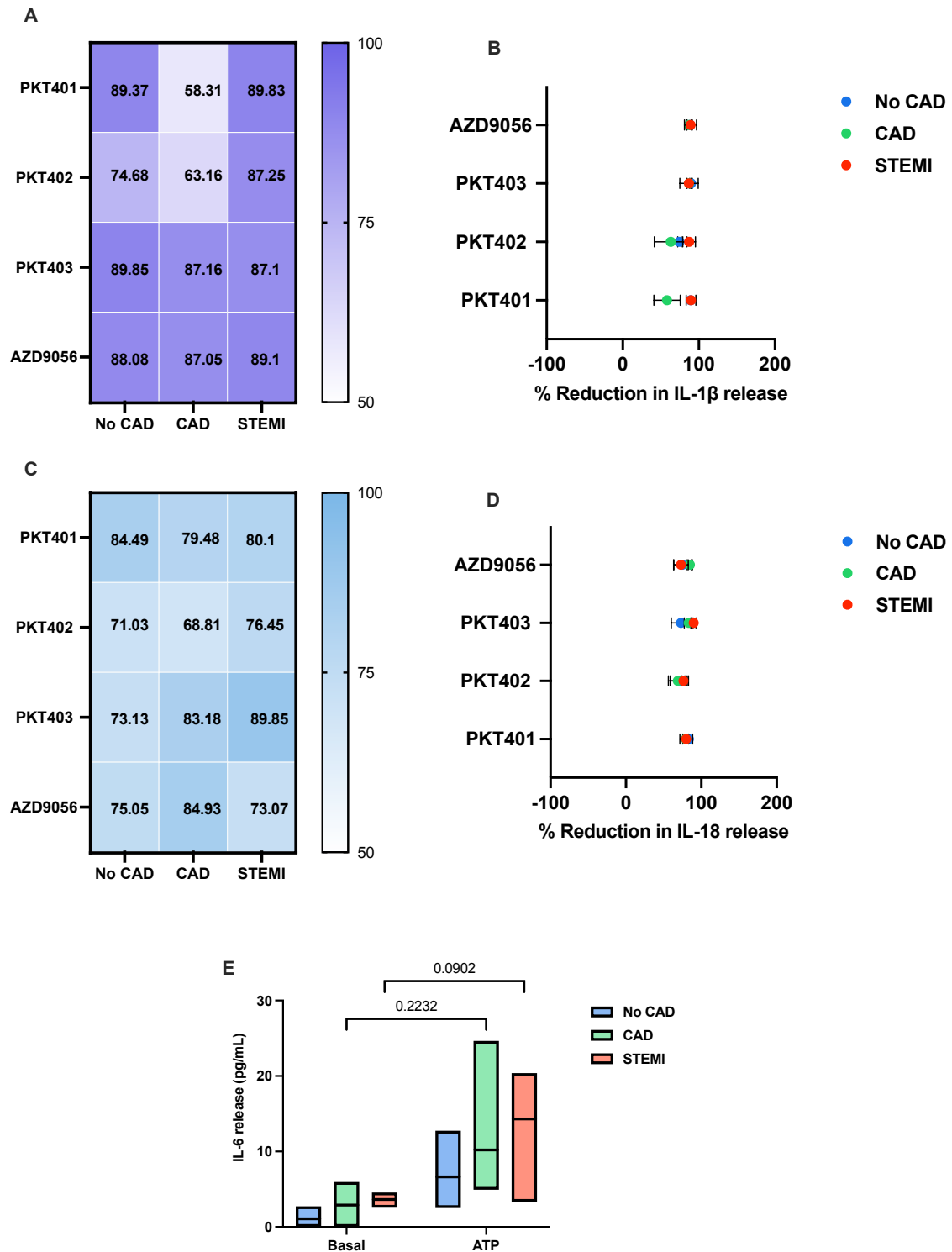
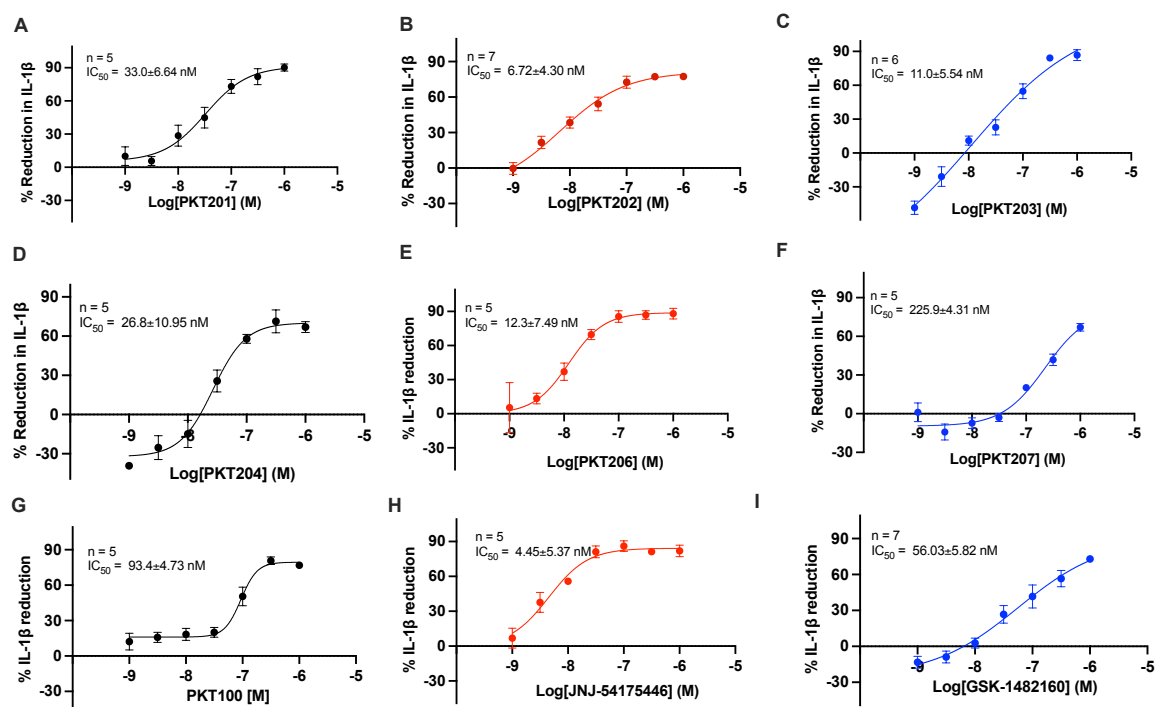
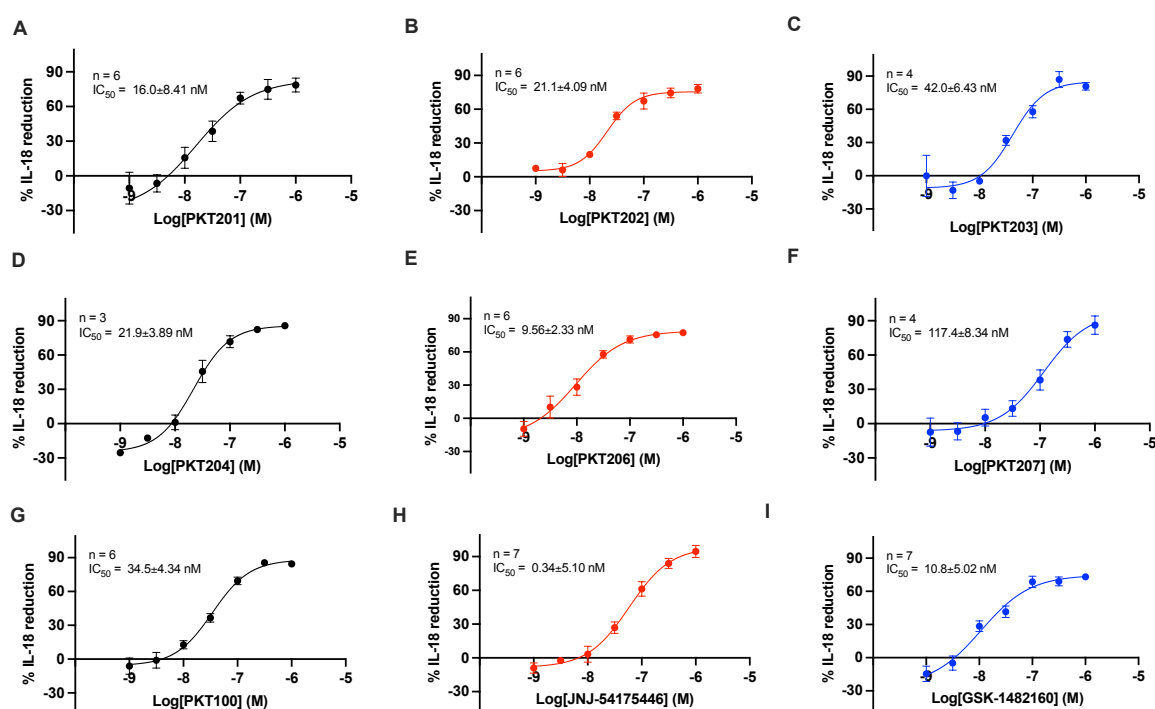


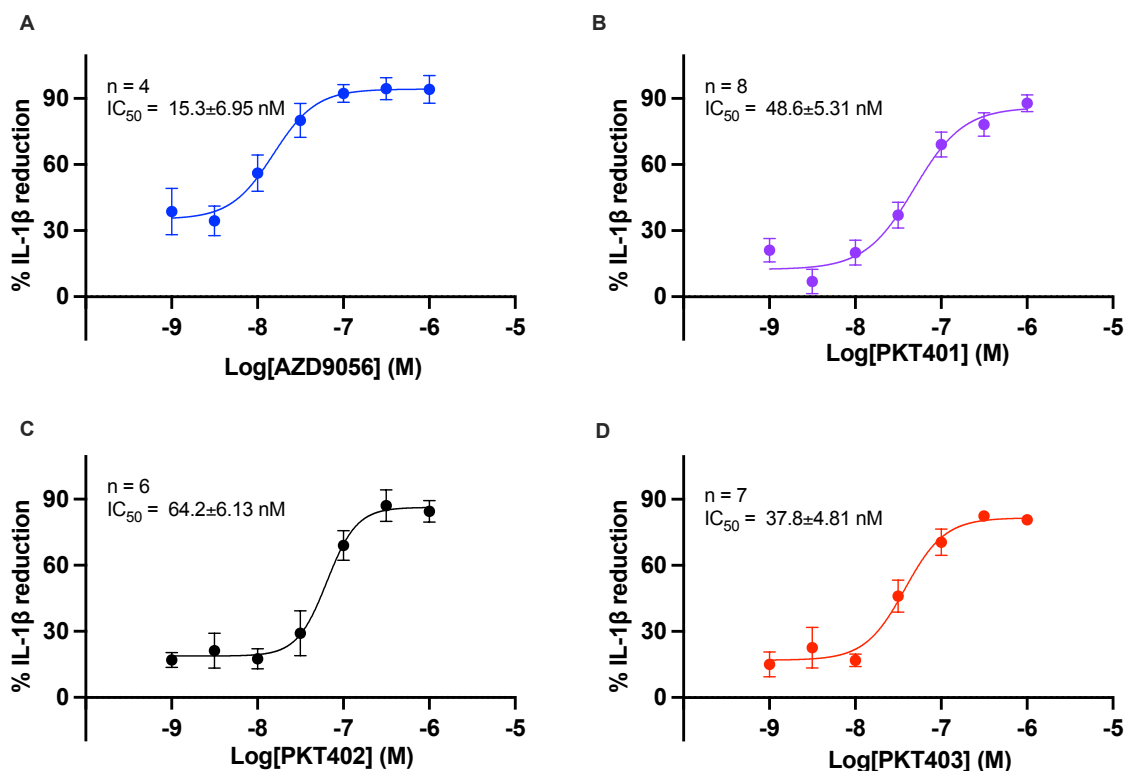
Figure 3. Bootstrap analysis finds potent inhibition of IL-1 β and IL-18 in monocytes derived from patients with no CAD, CAD, and STEMI. A) Heat map demonstrating the percentage reduction in IL-1 β elicited after BzATP stimulation in patients with no CAD, CAD, and STEMI. **B)** Visual representation of the percentage reduction in IL-1 β elicited in patients with no CAD, CAD, and STEMI with standard error mean bars to show variability) Heat map demonstrating the percentage reduction in IL-18 in patients with no CAD, CAD, and STEMI. **D)** Visual representation of the percentage reduction in IL-18 elicited in patients with no CAD, CAD, and STEMI with standard error mean bars to show variability. **E)** Concentration of IL-6 secretion from monocytes under basal and BzATP stimulated conditions in no CAD, CAD, and STEMI groups. Values are expressed as mean $\pm$ SD.



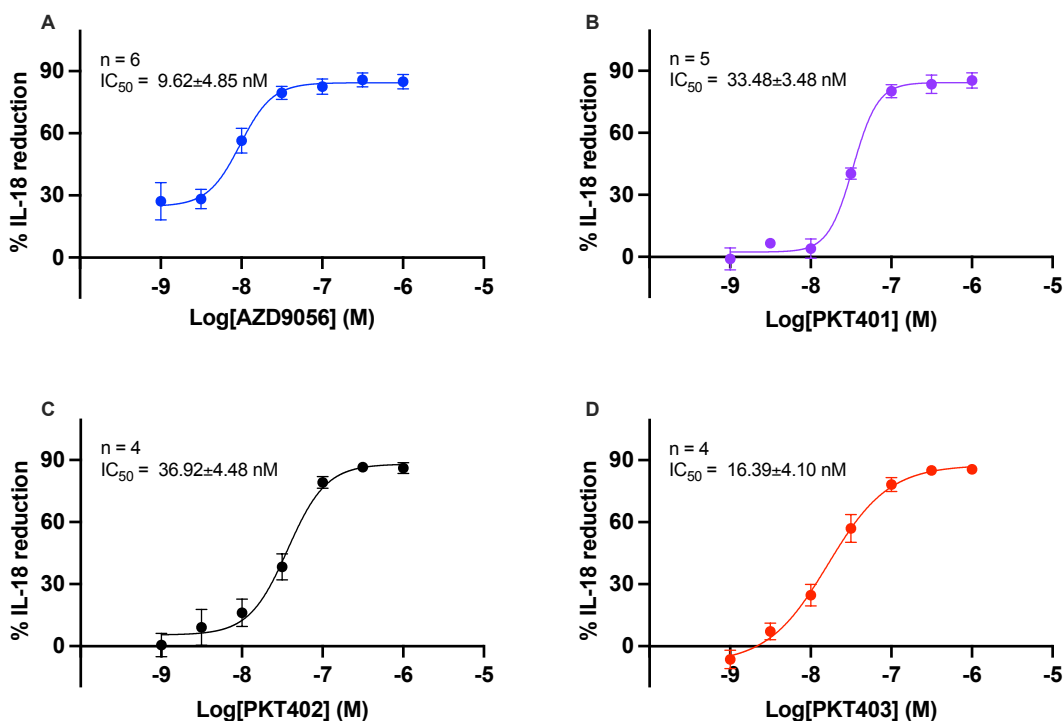
Supplementary Figure 1. Log concentration-response curves for IL-1 β release assays for the cyanoguanidine-adamantane series and benchmarking compounds. A) PKT201 (n = 5), B) PKT202 (n = 7), C) PKT203 (n = 6), D) PKT204 (n = 3), E) PKT206 (n = 5), F) PKT207 (n = 5), G) PKT100 (n = 5), H) JNJ-54175446 (n = 7), I) GSK-1482160 (n = 5).



Supplementary Figure 2. Log concentration-response curves for IL-18 release assays for the cyanoguanidine-adamantane series and benchmarking compounds. A) PKT201 (n = 6), B) PKT202 (n = 6), C) PKT203 (n = 4), D) PKT204 (n = 3), E) PKT206 (n = 6), F) PKT207 (n = 4), G) PKT100 (n = 7), H) JNJ-54175446 (n = 7), I) GSK-1482160 (n = 7). Values are expressed as mean $\pm$ SEM.



Supplementary Figure 3. Log concentration-response curves for IL-1 β release assay for the adamantane amide series and benchmarking compound. A) AZD9056 (n = 4), B) PKT401 (n = 8), C) PKT402 (n = 6), D) PKT403 (n = 7). Values are expressed as mean $\pm$ SEM.



Supplementary Figure 4. Log concentration-response curves for IL-18 release assay for the adamantane amide series and benchmarking compound. A) AZD9056 (n = 6), B) PKT401 (n = 5), C) PKT402 (n = 4), D) PKT403 (n = 4). Values are expressed as mean $\pm$ SEM.

Supplementary Table 1. Key clinical and demographic characteristics of Cohort 1 used in patient-derived monocyte stimulation assay in Figures 1.

	no CAD (CACS=0)	CAD (CACS>0)	STEMI
Participants (n)	21	34	24
CACS, y-median (IQR)	0	93.5±261	-
Age, y-median (IQR)	52±16	68±12.8	62±17.3
Female – no. (%)	16(76.2)	11(32.3)	2(8.3)
Hypertension – no. (%)	6(28.6)	20(58.8)	10(41.7)
Diabetes – no. (%)	1(4.8)	3(8.8)	3(12.5)
Hypercholesterolemia - no. (%)	12(57.1)	23(67.6)	10(41.7)
Significant smoking history– no. (%)	9(42.9)	14(41.2)	15(62.5)
Statin – no. (%)	7(33.3)	23(67.6)	4(16.7)
Aspirin - no. (%)	0(0)	5(14.7)	4(16.7)
Anti-hypertensives – no. (%)	7(33.3)	22(64.7)	-

Supplementary Table 3. Key clinical and demographic characteristics of cohort 3 used in patient-derived monocyte stimulation assay in Figures 3.

	no CAD (CACS=0)	CAD (CACS>0)	STEMI
Participants (n)	7	8	3
CT Calcium Score, y- median (IQR)	0	84.5 ± 237.75	-
Age, y-median (IQR)	59.0 ± 10.0	67.0 ± 10.75	58.0 ± 13.5
Female – no. (%)	3(42.9)	2(40.0)	0(0)
Hypertension – no. (%)	0(0)	0(0)	2(66.6)
Diabetes – no. (%)	0(0)	0(0)	0(0)
Hyperlipidemia – no. (%)	0(0)	0(0)	2(66.6)
Significant smoking history– no. (%)	2(28.6)	4(50.0)	0(0)
Statin – no. (%)	1(14.3)	2(40.0)	0(0)

Supplementary Table 2. Key clinical and demographic characteristics of Cohort 2 used in patient-derived monocyte stimulation assay in Figures 2.

	no CAD (CACS=0)	CAD (CACS>0)
Participants (n)	12	15
CT Calcium Score, y-median (IQR)	0	73.0 ± 120.0
Age, y-median (IQR)	56.0 ± 14.3	62.0 ± 9.0
Female – no. (%)	7(58.3)	2(13.3)
Hypertension – no. (%)	1(8.33)	1(6.66)
Diabetes – no. (%)	0(0)	0(0)
Hyperlipidemia – no. (%)	0(0)	1(6.66)
Significant smoking history– no. (%)	4(25.0))	5(33.3)
Statin – no. (%)	2(16.7)	4(26.6)

Chapter 7

Discussion

The persistence of cardiovascular disease (CVD) as the leading cause of mortality worldwide, despite the effective implementation of pharmacotherapeutic strategies, (1) underscores the presence of unresolved biological mechanisms that await discovery. Beyond traditional risk factors, inflammation has emerged as a crucial ungoverned contributor to the onset and advancement of CVD. (2) Particularly in the context of coronary artery disease (CAD), the formation of atherosclerotic plaque has evolved beyond its initial characterisation as a solely lipid-driven disease to encompass a network of complex interactions between innate and adaptive immune systems. (3,4) This broader perspective underscores the therapeutic potential of immunomodulation to enhance primary prevention efforts to reduce the global incidence of mortality from CVD. Thus, the primary objective of this thesis was to explore P2X purinoceptor 7 (P2X7R)-dependent inflammation as a contributor and therapeutic target to CVD. In short, this thesis aimed to evaluate whether P2X7R antagonism could attenuate inflammation and improve preclinical outcomes in murine models of CVD.

My work in this thesis has built on the strong evidence from the CANTOS (Canakinumab Antiinflammatory Thrombosis Outcome Study), demonstrating the therapeutic benefits of neutralising interleukin-1 beta (IL-1 β) in reducing future cardiovascular events in a high-risk population with a previous history of myocardial infarction (MI) and residual inflammatory risk. (5) This benefit was achieved in addition to intensive statin therapy, where Canakinumab was demonstrated to improve cardiovascular outcomes independent of lipid modification. (5) Despite these promising results, the modest effect size and increased risk of bacterial sepsis limited the clinical translation of Canakinumab for CAD. Building upon these observations, several secondary studies aimed to investigate whether the effectiveness of IL-1 β neutralisation was constrained by other factors. (6,7) Central to the core of this thesis is the observation that while interleukin-6 (IL-6) is reduced downstream of IL-1 β , interleukin-18 (IL-18) persists as a significant source of inflammation. (7) This evidence indicated that targeting proteins upstream of these cytokines may offer broader immune modulation and greater therapeutic benefits. Given these insights, an unmet need for immunomodulatory compounds capable of safely and effectively targeting the inflammatory contribution to CVD must be identified.

In this thesis, I have confirmed that P2X7R is a critical source of IL-1 β and IL-18, thereby acting as a potential pathogenic mediator in the development of CVD. Its activation in response to high concentrations of endogenous adenosine-5'-triphosphate (ATP), primarily due to tissue injury, has made it a valid candidate to target inflammation in CVD beyond the boundaries of the host immune defence. (8–10) This approach offers several advantages over traditional anti-inflammatories, particularly regarding the improved safety profiles of this class of compounds. Targeting these pathways with PKT100, the first-generation P2X7R antagonist, was shown to have favourable benefits against plaque instability, MI, pulmonary hypertension, (11) and obesity-driven heart failure with preserved ejection fraction (HFpEF). Previous P2X7R antagonists have mostly undergone evaluation in non-cardiovascular conditions such as rheumatoid arthritis (RA) and Crohn's disease,

both of which carry a substantial autoimmune-dependent pathogenic mechanism compared to CAD. (12–14) Under these circumstances, demonstrating efficacy on top of classical immunosuppressants has led to unfavourable clinical trial outcomes. Given these findings, a comprehensive drug development strategy has been assembled to expedite human trials with P2X7R antagonists. The target population for these human studies will be participants with computed tomography (CT)-defined CAD before the first events occur. The work presented in this thesis has laid the foundations for this strategy, which has garnered substantial interest from commercialisation and pharmaceutical companies. Based on the conclusions of the previous chapters, the next section will explore the significant findings and future directions of this project, highlighting the importance of this work in association with the literature.

P2X7R expression on a subset of classical and non-classical monocytes and CD1c⁺ DCs that closely correlate to CACS burden

Current diagnostic strategies for the detection of CAD are restricted to patients who are considered high risk for CAD based on traditional risk algorithms. (15) However, these current guidelines fail to capture patients in the subclinical atherosclerosis phase, where most patients may be asymptomatic or considered low-risk. (15) This includes 25% of first-time MI patients who present with ST-elevated MI (STEMI) despite no traditional risk factors. (16) In later stages, C-reactive protein (CRP) is considered an independent predictor of cardiovascular events. (17) Despite this, several limitations are associated with its use as a diagnostic marker in CAD. CRP demonstrates no causality in atherosclerosis and is synthesised in response to IL-6, making it largely non-specific and a marker of generalised inflammation. (18,19) The movement towards the detection of more specific markers now relies on high dimensional data such as proteomic and single-cell transcriptomics analysis of tissues, cells and plasma to find novel biosignatures indicative of disease status. Cytometry by time-of-flight (CyTOF) was utilised to evaluate the cellular proteome of immune cells at a single-cell level. These methods overcome the previous limitations of traditional flow cytometry through metal-tagging antibodies with elemental isotopes. This technique allows for the most comprehensive map of the immune system to be formed, using key markers to identify heterogeneous immune populations.

Peripheral blood mononuclear cells (PBMCs) from patients with computed tomography (CT)-defined CAD showed enriched expression of the P2X7R across classical and non-classical monocytes, CD141⁺ and CD1c⁺ myeloid dendritic cells (mDCs), and lesser populations such as B, natural killer (NK) cells, and plasmacytoid dendritic cells (pDCs). Together with comprehensive atherosclerotic plaque phenotyping, heightened expression of P2X7R was demonstrated on classical and non-classical monocytes and CD1c⁺ mDCs that closely correlate with coronary atherosclerotic burden as measured by coronary artery calcium score (CACS) and soft plaque score (SPS). The implications of these findings are two-fold. In the first instance, the discovery of heightened P2X7R expression on immune cells that share a close association with CACS burden and SPS suggests the biological relevance of P2X7R in atherosclerosis. In the second instance, the heightened expression of P2X7R across these immune populations could be used as a marker in atherosclerotic plaque activity or burden. Under

these circumstances, validation of this signature could be used in companion diagnostics in human trials to determine patient selection and early treatment efficacy particularly relevant for drugs targeting inflammasome-related pathways. In both these applications, using P2X7R intensity as a marker of atherosclerotic plaque progression or regression would fill an unmet need in cardiovascular disease management.

Despite this, more needs to be known about the function of P2X7R in these immune cells in atherosclerosis to understand its utility better. Classical monocytes constitute the largest subset of the total monocyte populations and are important mediators of lesion development. Unlike their non-classical counterparts, classical monocytes are demonstrated to transmigrate vascular walls, where they diversify into macrophages and play a crucial role in lipid transport and eventual lesion formation. (20) Although the P2X7R was known to be abundant in this population, the heightened intensity in CAD may imply the crucial role of this pathway in plaque destabilisation. This novel finding has provided more evidence to support the clinical development of a P2X7R antagonist for CAD. Apart from classical monocytes, P2X7R intensity was also increased in non-classical monocytes. These populations were traditionally characterised as anti-inflammatory monocytes due to their limited ability to undertake phagocytosis. However, recent evidence has challenged this notion and demonstrated that non-classical monocytes are the dominant inflammatory monocytes. (21,22) In atherosclerosis, these populations are characterised as the 'patrolling' immune cells, where their residence in the vasculature protects against endothelial dysregulation. (23,24)

However, non-classical monocytes have been shown to play a pathogenic role in atherosclerosis. (23,25) Previous studies have determined that non-classical monocytes produce greater superoxide and endothelial activation in patients with CAD. (23) These functions are hypothesised to be linked to the advancement of atherosclerotic lesions. However, its critical relation to the P2X7R in these populations still needs to be addressed. Under basic fundamental hypotheses, the P2X7R will likely modulate cytokine secretion in these populations. This will form the foundation of newly proposed projects related to the P2X7R in atherosclerosis. Outside of the realm of inflammation, previous work has demonstrated that P2X7R inhibition with PKT100 could attenuate oxLDL-induced superoxide production in endothelial cells. (26) This work, which is complementary to the investigations in this thesis, utilised endothelial-colony-forming cells that spontaneously sprout from the PBMCs. This study demonstrated that participants with heightened susceptibility to CAD demonstrate higher superoxide production. (26) The efficacy of PKT100 was compared to Colchicine, where PKT100 demonstrated an enhanced therapeutic benefit above Colchicine on attenuation of mitochondrial reactive oxygen species generation. (26) These results support the utility and development of a P2X7R antagonist for the treatment of CAD.

In response to these findings, the extent of P2X7R contribution in CAD has been further evaluated. To pair with the CyTOF data, an ex vivo whole blood stimulation assay was conducted to compare patients with CAD, STEMI,

and without disease side-by-side. The purpose of this work will be to evaluate the diversification of immune populations with CyTOF under baseline and 2'(3')-O-(4-Benzoylbenzoyl) adenosine 5'-triphosphate (BzATP)-stimulated conditions. Substantial literature has demonstrated that the standard P2X7R isoform B, a truncated version of the full-length receptor isoform A, is most commonly expressed in tissues. (27,28) This receptor is relatively inactive as the retention of a stop codon between intron 10 and 11 results in the formation of a receptor with a shortened cytoplasmic tail. (27–29) This modification may result in a receptor with reduced pore-forming capabilities. Comparatively, the P2X7RA isoform harbours the full ability to secrete cytokines, as is characterised by the P2X7R. (27) Interestingly, the heterotrimerisation of P2X7R A and B isoforms results in a receptor with a heightened affinity for ethidium uptake. (27) Two antibodies, will be used, one targeted to the extracellular loop and a second for the C-terminus domain, to investigate potential differences in the expression of these isoforms. Additionally, the collection of paired plasma samples will be used to identify the cellular secretome associated with P2X7R activation. The combination of these two techniques will better equip us to answer questions about the contribution of these immune populations to atherosclerosis and offers a unique opportunity to evaluate P2X7R as a potential biomarker for CAD.

P2X7R expression on a subset of classical and CD1c⁺ DCs that closely correlate with STEMI and CRP

In the case of STEMI, atherosclerotic lesions rupture in response to plaque weakening and increased biomechanical stress, which leads to embolism, thrombosis, and a substantial reduction of blood to the myocardium. (30,31) The immediate unmet needs of this population differ from those with stable disease before first-time events. In fact, the urgent requirement for revascularisation in the appropriate timeframe takes the greatest priority, followed by evidence-based intensive pharmacotherapies known to improve outcomes after MI. (16) After this event, this population presents with an increased risk for future cardiovascular events, which was determined in the CANTOS to be partly due to the residual inflammatory risk after MI. (5) This population may also greatly benefit from safer immunomodulatory strategies to improve survival outcomes. While there is an immediate need for diagnostic and therapeutic approaches in patients with stable CAD, the MI cohort allows for the prediction of markers associated with recurrent events; they are also a valuable comparison to stable CAD patients. As part of this chapter, it was shown that classical monocytes and CD1c⁺ mDCs demonstrate reduced P2X7R intensity in STEMI participants. Interestingly, the reduced intensity of P2X7R across these populations was associated with heightened CRP concentrations measured from the plasma.

Previous studies have shown that the soluble P2X7R is associated with disease severity. (32–34) Particularly, in patients with COVID-19, heightened levels of soluble P2X7R detected in plasma were associated with elevated CRP and poorer outcomes, such as mortality and hospitalisation. (32,33) These results were previously replicated in patients after MI and demonstrated that soluble P2X7R concentrations were strongly associated with CRP concentrations. (34) The above values were reported by those with infections. (34) This might corroborate the findings from the classical monocytes and CD1c⁺ mDCs in the STEMI population. Since it was shown that the proportion of these cells that expressed the P2X7R was uniform for classical monocytes and CD1c⁺ mDCs

between STEMI and no CAD groups, the reduction in P2X7R intensity is not attributable to apoptosis. Thus, this data is in agreement with previous literature, which suggests that activation of the P2X7R increases microvesicles shedding containing soluble P2X7R and mature IL-1 β from dendritic cells. (35) In this study, microvesicles shedding could be downregulated after treatment with the P2X7R antagonist KN-62. (35) This mechanism has been demonstrated to be modulated by P2X7R activation of metalloproteinase-2, which in turn is cleaved by metalloproteinase-2. (36) However, while this feedback loop is hypothesised to fine-tune P2X7R function, the biological and immunological consequences of this mechanism in MI have not been elucidated.

Under these circumstances, the discoveries from this cohort have evoked a number of questions that will drive future work. These patients will be included in the planned project above but have provoked an interest in exosome and microvesicles characterisation in patients with CAD. While previous work in the team has used Fourier-transform infrared technology for high-throughput evaluation of isolated exosomes from CAD participants, greater depth can be included in the assessment of the STEMI population. Moreover, evaluating the intravesicular contents may better discriminate the unique signature associated with the pathogenesis of atherosclerosis. As part of this ongoing study, an in-depth evaluation of the immune populations in this cohort will be continued. The data will also be compared with data from a 3-day murine MI model, where PKT100 has been evaluated as a pharmacotherapeutic against adverse remodelling. The PBMCs and hearts of these mice have been prepared for immunophenotyping to allow better discrimination of the effects of P2X7R antagonism after MI.

P2X7R-dependent BzATP cytokine release is upregulated from monocytes derived from patients with CAD.

Exploring the P2X7R in these immune populations led to the subsequent novel discovery in patients with CAD. For the first time, it was demonstrated that monocytes derived from patients with CAD demonstrated an increased inflammatory burden associated with heightened IL-1 β release after BzATP stimulation. This enhanced inflammatory profile was associated with increases in CACS, indicating that the inflammatory signature of PBMCs may reflect the burden at the level of the plaque and may be P2X7R-dependent. Combined with the findings above, this suggested that the P2X7R may be involved in atherosclerotic plaque progression, and thus, targeting the P2X7R could be beneficial for the treatment of CAD. However, these results have greater implications beyond the use of P2X7R as a therapeutic target. Human trials with cohorts with CAD have utilised CRP as a criterion for patient selection based on their supposed inflammatory status. (5,37) Fluctuations in plasma CRP has accompanied outcome data to demonstrate early therapeutic efficacy. Including surrogate markers in clinical studies can reduce the time required until approval and expedite decisions to proceed or terminate clinical trials.(38) This approach has been typically applied to oncology, where trials require long-term follow-up to demonstrate clinical efficacy. (39) While this does not replace the requirement for robust, long-term trials, it may continue to support the biological relevance of treating the disease. It was hypothesised that P2X7R-dependent IL-1 β release could be a surrogate marker to demonstrate clinical efficacy in cardiovascular trials

with P2X7R antagonists. The causal relationship between atherosclerosis and cytokine IL-1 β supports the utility of this pathway as an endpoint.

This same idea has been applied to several human trials with P2X7R antagonists. (13,40,41) Oral administration of AZD9056 was demonstrated to reduce ATP-induced IL-1 β release by above 90% in healthy participants. (13) Although this was not translated into positive clinical trial outcomes in a cohort with RA, there are certain complexities around the treatment of RA compared to participants with CAD. For instance, it is a systemic autoimmune disorder currently characterised by the presence or absence of anti-citrullinated protein antibodies (ACPAs). (42) ACPAs are a class of autoantibodies resulting from the post-translational modification of arginine residues into citrulline. (42) The alternations in their structure result in autorecognition from T cells, which activate macrophages to promote inflammation. (43,44) Genetic polymorphisms in RA contribute 30 to 60% of the overall risk, with human leukocyte antigen class II alleles demonstrating the greatest risk, particularly in cohorts with ACPA-positive RA. (45) Fibrosis-driven pathways have been highlighted as contributors to RA. (46,47) Croft et al. characterised a fibroblast-activation protein expressing fibroblast population upregulated in the synovial lining. (48) Deleting this distinct fibroblast population has been validated to suppress inflammation and tissue erosion in a mouse arthritis model. (48) Therefore, while RA is characterised primarily as an autoimmune disorder, the paracrine relationship between immune cells and mesenchymal populations promotes deleterious clinical manifestation, which is beyond the benefits of a P2X7R antagonist. However, recent clinical trials with P2X7R antagonist JNJ-54175446 in a population with mood disorders showed modest benefits with substantial reductions in ex-vivo patient-derived ATP-induced IL-1 β release, (41) demonstrating this concept.

The final utility of this assay would be its use as a companion diagnostic in human trials to identify participants who are more likely to respond to treatment. This relates to the absolute and relative reductions in IL-1 β demonstrated in this chapter. This can only be conducted in targets with a well-known biological background, (49) such as the P2X7R. This has also been largely applied to the oncology field, where the idea of personalised medicine is notorious since the underlying pathogenesis of cancer varies drastically from person to person. (50) This framework can also be applied to the STEMI participants who demonstrated greater increases in BzATP-induced IL-1 β release. While the involvement of IL-6 and CRP in the acute setting, where their concentrations rise significantly after MI, is better established, the discovery of increased P2X7R-dependent IL-1 β release from monocytes alongside heightened intracellular burden represents a novel finding. These results also indicate the relevance of this pathway in MI and the potential therapeutic value of drugs of this class, together with established evidenced-based interventions. However, several questions remain relevant to this project that now require addressing. Particularly, an intended focus will be placed on the genetic variance in these patients, particularly the single-nucleotide polymorphisms related to the P2X7R and NOD- LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome. These polymorphisms will be evaluated to better disentangle heightened IL-1 β secretion values independently of disease status and how responsiveness to PKT100 is

affected. This also ties back to some initial questions posed above about isoform differences in the acute and chronic state and the biological implications of P2X7R microvesicles shedding.

P2X7R-antagonism stabilises plaque in a mouse model of plaque instability

The improved benefits in mortality and recurrent events associated with Canakinumab have resulted in a renewed interest in anti-inflammatories for CAD. (5) While human trials with Colchicine improved cardiovascular outcomes beyond what was demonstrated with Canakinumab, its translation was limited due to safety concerns around long-term treatment. (51) Based on the initial findings, P2X7R antagonism as a therapeutic strategy was explored in atherosclerotic. In this study that utilised a novel murine model of plaque instability, for the first time it was demonstrated that PKT100 improved features associated with plaque stabilisation, such as neovascularisation, intraplaque haemorrhage, lipid-rich necrotic core size, and mature collagen deposition. The results from this study support the hypothesis and demonstrate that P2X7R antagonism is a valid strategy for the management of CAD. The implications of these results are beyond the benefits of this thesis and may result in the clinical development of a P2X7R antagonist for CAD.

Based on the evidence from Chapter 3, it was clear that P2X7R had a fundamental role in atherosclerotic plaque development. Previous studies in P2X7R^{-/-} mice showed that plaques were attenuated in atheroprone mice compared to their wild-type counterparts. (52,53) Similar results were demonstrated with the NLRP3 inflammasome inhibitor MCC950 in a model of diabetes-associated atherosclerosis. (54) However, the efficacy of a P2X7R antagonist had never been evaluated. These results do not completely reflect the results of the initial findings in P2X7R^{-/-} mice. (52,53) In fact, the conclusions of these studies showed plaque attenuation associated with P2X7R depletion. (52,53) This seemed to be associated with a reduced uptake of lipids in macrophages, suggesting that in the absence of P2X7R, mice were less prone to foam cell formation. (52) This study made the case for P2X7R-dependent inflammation as a potential causal pathway in atherosclerosis clear. However, there was no attenuation of atherosclerotic lesions in stable or unstable segments. Instead, the benefits of P2X7R antagonism were clearly in the features associated with unstable lesion formation. For example, there was clear benefits in the reduction of the lipid-rich necrotic core, otherwise known as soft plaque, which has been hypothesised to predict atherosclerotic plaque rupture. (55–57) These regions are filled with the deposition of cellular membranes, particularly erythrocytes and macrophages prone to necroptosis. (55,57,58) This mechanism is proposed to be associated with intraplaque haemorrhage, which are believed to result from the rupture of small capillaries in atherosclerotic plaque. (59) These results demonstrated that P2X7R antagonism inhibits all three processes, including 1) neovascularisation, 2) intraplaque haemorrhage, and 3) expansion of the lipid-rich necrotic core. These results demonstrate the stabilisation of atherosclerotic plaque with PKT100.

While the disparity in these findings has not been addressed, there are several hypotheses to explain these unexpected results. Most simply, P2X7R^{-/-} mice demonstrate global knockdown with substantial reductions in IL-1 β from bone-derived monocytes. (52,53) This is associated with a lifelong reduction of inflammation and

does not account for modifications to other receptors, such as P2X purinoceptor 4, which shares a close sequence homology with the P2X7R. More complicated is the efficacy of structural motifs at the P2X7R, alongside intricacies on the pharmacokinetic properties of individual compounds. Concerning the first point, P2X7R antagonists have demonstrated substantial variability in potency between rodent and human receptors. (60) For instance, the development of PKT100 is based on a prior adamantane benzamide structure development by AstraZeneca. (61,62) The initial structure demonstrated single nanomolar potency but was hindered by poor metabolic stability and substantial interspecies variability. (61,62) Bioisosteric substitution to the adamantane moiety improved the metabolic stability of the compound but also enhanced the potency at the mouse P2X7R. (62) However, compared to humans, PKT100 still demonstrates a six-fold reduction in the potency at the rat receptor. While the concentrations selected for preclinical studies have been adjusted for this variation, it is plausible that the concentration may fall below the thresholds required to achieve adequate and persistent IL-1 β release. (40) To determine whether these thresholds were reached with PKT100, PBMCs collected from the mice in these studies will be used to measure IL-1 β release in response to ATP. Overall, it is hypothesised that PKT100 should reduce IL-1 β release from monocytes.

To the second point, PKT100 demonstrate adequate bioavailability and half-life to be translated to humans. However, in mice, the half-life was reduced to 11 minutes. (62) For this reason, systemic delivery of PKT100 in an osmotic minipump was utilised instead of oral delivery. With the recent development of the P2X7R antagonist presented in the thesis, emphasis has been placed on the development of amounts with greater half-life than previous compounds. Under these circumstances, a side-by-side evaluation can be completed to understand whether P2X7R is involved in plaque attenuation. Despite these unknowns, recent studies with MCC950 and IL-1 β monoclonal antibodies have failed to demonstrate the same therapeutic benefit. (63) In these studies, MCC950 alone failed to elicit plaque stabilisation features or effects on systemic inflammation. (63) Comparatively, the IL-1 β monoclonal antibody reduced systemic inflammation but did not affect overall plaque stabilisation. (63) Under these circumstances, further evidence is required to understand how PKT100 can elicit a therapeutic benefit in plaque stabilisation.

These future studies are promising and aim to address several of the questions posed above. Much like the patients with CAD, fixed PBMCs collected from mice with tandem stenosis will be used to address the effect of P2X7R antagonism on the immune system. A 34-metal tagged antibody panel has been designed to characterise the circulating immune populations in this study. Of these antibodies, two antibodies will be used to detect the P2X7R. One of these antibodies APR-008, which targets the extracellular domain will detect both the P2X7RA and B isoforms as described above, whereas APR-004 targets the cytoplasmic domain. Therefore, this antibody will only detect the P2X7RA isoform, allowing for precise comparison of P2X7R isoforms under inflammatory conditions. PBMCs from these mice will be evaluated side-by-side with PBMCs from mice that have undergone a 3-day MI model. These initial discrepancies in P2X7R intensity that were found in human populations will be evaluated with the aim to elucidate the effects of P2X7R antagonism in atherosclerotic disease. As these cells

were fixed before frozen, a large panel of intracellular markers to address the activation of several of these inflammatory-driven pathways will be used. In combination, atherosclerotic plaques from unstable and stable lesions from the tandem stenosis model has been collected. Image mass cytometry will be used to provide the most extensive characterisation of unstable plaque in mice with a detailed comparison to human atherosclerotic vessels collected from patients undergoing atherectomy. As part of the initial study, the diversification and differentiation of immune populations will be addressed from unstable lesions from the murine model, focusing on anatomical landmarks. This will be overlaid with a comprehensive map of atomic force microscopy measurements to understand whether PKT100 increases the biomechanical stiffness of atherosclerotic plaques.

P2X7R-antagonism as an immunomodulatory strategy in cardiometabolic disease associated with obesity

The earlier work presented in patients with heightened P2X7R-dependent inflammation pointed to the biological relevance of this pathway in CAD. However, patients with HFpEF present with many of the same risk factors as those with CAD without sufficient benefits from available pharmacotherapeutics. (64,65) Recent literature focused on the benefits of sodium-glucose co-reuptake inhibitors in this cohort, which have primarily demonstrated benefits through improvements in metabolic parameters such as weight and glucose management. (66–69) These compounds are determined to have therapeutic benefits via inhibition of glucose absorption and reduction in metabolic parameters. (66) Preclinical studies in mice demonstrated an alternative therapeutic pathway for these compounds via inflammation. (70) However, these effects were indirect and reliant on the depletion of calcium. The reduction in inflammation was secondary to these effects and was shown to be mediated through NLRP3 inflammasome downregulation. (70) Based on these results, this study explored whether the benefits of an anti-inflammatory treatment could be achieved without improvements in metabolic parameters. This study demonstrated that P2X7R antagonism may be an effective therapeutic strategy in obesity-driven HFpEF, independent of reducing lean body mass or glucose tolerance. The most profound differences were in the diastolic and myocardial deformation parameters assessed by echocardiography, demonstrating the preservation of diastolic function associated with PKT100.

Further evaluation of this model showed that reduced interstitial fibrosis in the myocardium might mediate these functional benefits. While the P2X7R is ubiquitously expressed, its function on immune cells is better characterised than any other population. There was an abundance of P2X7R expression from monocytes in the non-ischemic dilated heart, while the expression in fibroblasts was negligible. These early findings provide some evidence that the potential benefit of a P2X7R antagonist in CVD may be immunomodulatory. Bulk transcriptional analysis demonstrated substantial reductions in pro-inflammatory and fibrotic pathways. This concomitant reduction in both pathways suggests that inflammation may be a crucial mediator in HFpEF pathogenesis. However, it is important to note that transcriptomics evaluation does not detect all genes associated with a cell, and better depth is required to capture lower-expressed sequences. This is particularly common for single-cell RNA sequencing, where the greater number of cells captured is often prioritised above

the sequence depth. P2X7R was not found in endothelial cells from this dataset; however, persistent evaluations have identified P2X7R expressed in endothelial colony forming cells from patients with CAD.

There are relatively few studies that have evaluated inflammatory pathways in HFpEF. Recent focus on the adaptive immune system identified T cells as early infiltrators in the myocardium in mice with HFpEF phenotype. (71) This was accompanied by a higher inflammatory burden mitigated in a T cell-deficient mouse. (72) Additionally, mice deficient in T cells demonstrated protection against diastolic dysfunction and cardiomyocyte hypertrophy. (71) In a separate study, loss of myeloid TREM2 was associated with increased ventricular hypertrophy and diastolic dysfunction. (72) Plasma evaluation of TREM2 demonstrated increased circulating concentrations of soluble TREM2 in patients with HFpEF, (72) which continues to suggest the importance of inflammatory drive pathways in the clinical presentation of the disease.

As part of the next study, this model will be completed in a two-hit model of HFpEF. This model recapitulates the clinical presentation of the condition much more closely, with a greater burden from hypertensive and obesity features. (73) It would also be imperative to include older and female mice in this study since this condition more often presents in older, post-menopausal women. (74) However, some inconveniences are associated with this study, primarily related to the requirement for aged, reproductively senescent mice. This study will increase the translational impact of a P2X7R antagonist, given that patients with HFpEF are more likely to also present concomitantly with atherosclerosis.

Development of a P2X7R antagonist for the treatment of CAD: Cyanoguanidine-adamantane series

The final chapter of this thesis explored two novel series of P2X7R antagonists aimed at reducing inflammation in CAD. Most commercially available P2X7R antagonists have been focused on enhancing compound permeability across the blood-brain barrier to target neuroinflammation due to the high abundance of P2X7R in microglia. However, peripheral restriction is preferable for the treatment of CVD, offering significant therapeutic benefits and fewer off-target effects. Moving from the first-generation P2X7R antagonist, the initial candidates that were developed were based on the cyanoguanidine motif synthesised by Abbott Laboratories. (75–78) These compounds demonstrated improved potency compared to other P2X7R antagonists with an adequate half-life for preclinical studies. In an attempt to further improve the half-life but retain the potency of this compound for human studies, the adamantane amide scaffold was included. (75) The cyanoguanidine-adamantane hybrids were potent P2X7R antagonists against IL-1 β and IL-18 in THP-1 cells. The least potent compound, PKT207, had the fluoride-for-hydrogen substitution on the adamantane bridgeheads, which was characteristic of PKT100.

Next, these compounds were ranked in the ex vivo patient-derived assay for candidate selection based on these initial results. The strategy was to incubate cells with a single concentration of P2X7R antagonists based on this series median half-maximal inhibitory potential in IL-1 β release assays. Moreover, several commercially

available benchmarking compounds were used to demonstrate the performance of the compounds compared to those previously evaluated in human trials. GSK-1482160 was selected based on its poor target engagement in human trials. (40) This compound was used as the minimum threshold requirement for effectiveness to reduce IL-1 β release. Comparatively, JNJ-54175446 was set as the gold standard for inflammatory inhibition. (41) JNJ-54175446 has previously been demonstrated to have sub-nanomolar potencies and a prolonged half-life compared to other P2X7R antagonists. (41,79,80) Two compounds, PKT202 and PKT206, which reached the necessary reductions in inflammation to warrant clinical translation. In fact, this series revealed several new findings that were not realised with the evaluation of the first-generation compounds. Firstly, it must be noted that it was selected to move from 100 μ M of BzATP to 1 mM to maximise the effect of IL-1 β release through the receptor. This may be reflected in some of the observable differences with the benefits of PKT100 in Chapter three.

Secondly, regardless of the potency established in vitro, several compounds, apart from PKT201, PKT207, and GSK-1482160, performed better across patients with STEMI than the other cohorts. There may be several reasons for this. STEMI participants may demonstrate a greater release of IL-1 β from monocytes than CAD participants and, thus, greater reductions in IL-1 β release. This is something that has been evaluated in depth, given the substantial resources required to assess these samples alongside each other for an accurate assessment of concentration. Since then, the development of high-throughput methods incorporating proximity ligation assay enables cost-effective, more timely evaluation of cytokines. The next plan is to evaluate supernatants from the same patients to demonstrate concentration variations between the cohorts. Another possibility, which may feedback to the initial hypothesis, is that P2X7R-dependent inflammation becomes more relevant in the acute setting. Strong biological data support this hypothesis, which relates back to the idea of the P2X7R being shed into circulation after MI. This would also help to explain the initial observations that BzATP-induced IL-1 β release is associated with CACS — a measure of the overall burden of atherosclerotic disease. Under these circumstances, this data supports the benefit of a P2X7R antagonist in CAD.

This chapter was concluded by discovering PKT206 as the lead candidate for clinical translation based on its efficacy in patient-derived monocytes. However, subsequent metabolic stability studies showed that all compounds were liable to rapid metabolism and clearance and, thus, would not be appropriate for clinical development. These compounds were quickly terminated, although future studies may consider these compounds for the detection of vascular inflammation. Their relatively short half-life coupled with enhanced potency may be suitable pharmacokinetic properties for the development of a P2X7R-specific positron emission tomography ligand, although this is beyond the scope of this thesis. As mentioned previously, the future of this project aims to characterise these patients at the genomic level. Polymorphisms associated with non-synonymous mutations of the P2X7R and NLRP3 will be evaluated coupled with IL-1 β and IL-18 release. As described below, this project was suitable for two commercialisation grants that have extended the work on this target.

Development of a P2X7R antagonist for the treatment of CAD: fluorinated adamantane-amide series

The final finding related to this thesis is the discovery of fluorinated adamantane-amide compounds. Although this study is the shortest, it has the greatest opportunity for clinical translation to patients with CAD. Ex vivo evaluation demonstrated the substantial reductions in IL-1 β release with AZD9056. This structural motif is based on the initial adamantane amide compounds evaluated by AstraZeneca. (61) Since then, most P2X7R antagonist developed have used this structure as a basis for their synthesis. bioisosteric fluoride-for-hydrogen substitution to the adamantane bridgeheads of AZD9056 improved the physiochemical properties of the compound for CAD. The outcome of this modification was a series of compounds with enhanced metabolic stability compared to the cyanoguanidine series. Particularly, the monofluoride compound was demonstrated to behave similarly to AZD9056, although it showed a reduction in metabolic stability in human liver microsomes. However, metabolic stability for this compound was heightened in rodent liver microsomes. Comparatively, the difluoride compound showed the poorest potency out of the series, with minor improvements in metabolic stability at the human receptor compared to the monofluoride compound. The trifluoride adamantane reminiscent of PKT100 showed a drop in potency but improved half-life beyond the values demonstrated for AZD9056 in human liver microsomes. The metabolic stability was reduced in rodents but was substantial compared to the previous series. This evidence determined that the difluoride compound PKT402 was unlikely to proceed to clinical development, but PKT401 and PKT403 showed promise.

Ex vivo evaluation of these compounds against CAD, STEMI, and No CAD monocytes demonstrated that PKT403 and AZD9056 demonstrated the greatest efficacy against BzATP-induced IL-1 β release. This did not surprise us as these compounds had performed relatively the same when assessed for potency in THP-1 cells. In comparison, IL-1 β reduction was lowest among patients with CAD, while the efficacy of PKT401 and PKT402 was retained among the other populations. However, it is worth noting that compared to the initial analysis with the cyanoguanidine-adamantane hybrids, a smaller number of patients has been included in this assessment. Based on these findings, further experiments are now underway to evaluate the efficacy of these compounds in a wider population. In STEMI participants, a consistent reduction across all compounds was found, independently of potency. This continues to reconfirm the biological relevance of this pathway after acute MI and suggests a potential utility of these compounds in this cohort.

This is also the first comprehensive assessment of IL-18 reduction in response to a P2X7R antagonist. Despite differences in the reduction of IL-1 β among compounds, it was observed that IL-18 is consistent across compounds and reaches close to maximal values in cohorts. While the maturation of both cytokines is modulated in part by the P2X7R, previous studies have demonstrated that their synthesis and release are not linear. (81,82) For example, while IL-18 has been shown to maintain a persistent concentration in myeloid cells despite no evidence of inflammation, IL-1 β has only been found in negligible amounts in healthy participants. (81,82) This may reflect its required role in the type 1 and 2 T helper cells differentiation and mediation of immunomodulation. (83)

This final chapter concludes that PKT401 and PKT403 are the most promising lead candidates from a series of adamantane-amide compounds targeting the P2X7R. These compounds have attracted substantial funding from commercialisation grants to develop them as a treatment for CAD and other cardiovascular disease states associated with sterile inflammation. The immediate next step for this project is to assess these compounds in toxicology and safety screening and rodent pharmacokinetic studies. The results of these experiments will determine which compound is used in a subsequent model of plaque instability. To align ourselves with the current requirements of pharmaceutical companies, the drug delivery route will be through daily oral or intraperitoneal administration. Moreover, these new compounds will be evaluated in a mouse model of pulmonary hypertension to complement the previous study with PKT100. In this model, mice treated with PKT100 demonstrated significant improvements in survival outcomes primarily associated with better right ventricular function measured with echocardiography. (11) These results were achieved independent of a reduction in right ventricular systolic pressure. (11) While current treatments in these cohorts aim to improve pulmonary hypertension, there are no available pharmacotherapeutics that target RV failure. Under these circumstances, these patients demonstrate a significant unmet need for compounds that target the right ventricle. Moreover, the recent success of these compounds, combined with increasing evidence for the role of P2X7R in CVD, has reinvigorated interest in this novel class of compounds. An established collaboration with two leading pharmaceutical giants has resulted in interest in rarer CVD conditions, such as arrhythmogenic right ventricular cardiomyopathy. This condition is relatively rare and is genetically determined through mutations in desmosome proteins such as plakophilin-2. (84) This collaboration will mainly evaluate P2X7R antagonism as a potential therapeutic strategy in this unique population to gain early access to clinical trials.

Conclusion

The demonstrated clinical benefits of IL-1 β neutralisation in CVD have unlocked avenues for targeting inflammation in CVD. However, the heightened susceptibility to sepsis and reduced effect size in clinical trials suggests the greater benefit of targeting inflammation upstream of these cytokines. It was shown that the P2X7R is a valid diagnostic and therapeutic pathway in CAD. Heightened expression of P2X7R in CD1c⁺ mDCs, nonclassical, and classical monocytes from participants with CT-defined CAD was associated with plaque destabilisation. After infarction, expression of P2X7R on CD1c⁺ mDCs and classical monocytes was downregulated. However, both CAD and STEMI populations demonstrated substantial increases in BzATP-induced IL-1 β release compared to those without CAD. This response could be diminished with a novel P2X7R antagonist PKT100. These results continue to support the biological relevance of this pathway in chronic and acute CVD. Treatment with PKT100 in a mouse model of plaque destabilisation resulted in plaque stabilisation, indicated by the reduction in the lipid-rich necrotic core, neovascularisation, intraplaque haemorrhage and increases in intraplaque collagen. These features indicate a movement towards plaque stabilisation, irrespective of plasma lipids. Mice treated with PKT100 in an obesity-driven HFpEF demonstrated preserved diastolic function linked with reduced interstitial fibrosis. P2X7R expression was abundant in monocytes and macrophages in the heart but relatively absent in non-immune cells such as fibroblasts and endothelial cells.

Gene expression changes were associated with a downregulation in inflammatory transcripts coding for IL-1 β and MCP-1. These results suggest a potential immunomodulatory benefit of PKT100 in HFpEF, independent of a direct effect of fibrotic pathways. These promising results lead to the development of two novel series of P2X7R antagonists. The cyanoguanidine-adamantane series was potent but lacked metabolic stability and thus was terminated. The development of the fluoride adamantane-amides was potent, with heightened metabolic stability appropriate for clinical development. Under these circumstances, this thesis developed a novel series of P2X7R antagonists to target the inflammatory contribution to CVD. While these findings have provided novel insights into the potential benefits of P2X7R antagonism in CVD, a greater number of questions have remained unanswered. However, the future directions of these projects have the capability to change cardiovascular medicine.

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