Doctor of Medicine

Telomerase Activation to reverse Immunosenescence in elderly patients with Acute Coronary Syndrome

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Abstract

Introduction:

Immune ageing is characterised by lymphopenia, expansion of pro-inflammatory T-lymphocytes and reduced telomere length. Acute coronary syndrome (ACS) evokes an intense inflammatory response and leads to accelerated immune ageing, reflected by an increase in $CD8^+$ terminally differentiated T lymphocytes ($CD8^+$ T_{EMRA}). While activation of telomerase enhances lymphocyte proliferation in-vitro and reverses tissue degeneration in aged mice, its potential benefit in patients recovering from ACS is unknown.

Methods:

This randomized, double-blinded controlled study aimed to evaluate the safety and efficacy of the oral telomerase activator TA-65 in 90 MI patients aged over of 65 years, which is the average onset age for immune ageing. Patients were randomised to either TA-65 (16 mg daily, n=45) or placebo (n=45) for 12 months. Immune phenotyping was performed by flow cytometry at baseline, 6 months and 12 months. The pre-defined primary endpoint was the proportion of $CD8^+$ T-lymphocytes which were $CD8^+$ T_{EMRA}, a marker of immune cell ageing. Secondary endpoints included high-sensitivity C-reactive protein (hsCRP) and adverse events. A linear mixed effects model was used to calculate the treatment effect.

Results:

The median age of participants was 71 years and 17% were women. Most patients received percutaneous coronary intervention (87%). The proportion of $CD8^+$ T_{EMRA} did not differ between the 2 treatment groups at 12 months, but increased significantly only in the placebo group (+2.2 %, 95% CI 0.14-4.24). Total adverse events were 30% lower in the treatment group (n=130 vs n=185).
High-sensitivity CRP was 62% lower in the TA-65 group compared to placebo (1.1 vs. 2.9 mg/L). There was a 14% increase in mean lymphocyte count in the TA-65 group (+240 cells/µl, 95% CI: 109 – 374), driven by significant increases from baseline in all major lymphocyte populations: $CD3^+$ (+14%), $CD4^+$ (+14%), $CD8^+$ T-lymphocytes (+14%), B-lymphocytes (+14%) and natural killer cells (+16%), while major lymphocyte populations did not increase in the placebo group.

Conclusion:

While TA-65 did not significantly alter $CD8^+$ T_{EMRA} ,it increased all major lymphocyte subsets and reduced hsCRP in elderly patients with MI at 12 months. TA-65 may be a novel agent to reduce inflammation and improve age-related decline in major lymphocyte populations after MI.

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Abbreviations

Abbreviation	Definition
ACS	Acute Coronary Syndrome
AE	Adverse Event
AR	Adverse Reaction
BP	Blood Pressure
CABG	Coronary Artery Bypass Graft
CAD	Coronary artery disease
CAG	Cycloastrogenol
CI	Chief Investigator
CMV	Cytomegalovirus
COPD	Chronic Obstructive Pulmonary Disease
CRF	Electronic Case Report Form
CVD	Cardiovascular disease
DMEC	Data monitoring and ethics committee
DNA	Deoxyribonucleic Acid
FACS	Fluorescence-activated cell sorting
HDL	High-density lipoprotein
hsCRP	high sensitivity C-Reactive Protein
IB	Investigator Brochure
IDMEC	Independent Data Monitoring and Ethics Committee
IFN	Interferon

IHD	Ischemic heart disease
IL	Interleukin
IMP	Investigational medicinal product
JCUH	James Cook University Hospital
LDL	Low-density lipoprotein
LV	Left ventricle
LVEF	Left ventricular ejection fraction
LTL	Leukocyte telomere length
MACE	Major adverse cardiovascular events
MHRA	Medicines and Healthcare products Regulatory Agency
MI	Myocardial Infarction
NCTU	Newcastle Clinical Trials Unit
NHS	National Health Service
NSTEMI	Non-ST segment elevation myocardial infarction
NT-proBNP	N-terminal pro-B-type natriuretic peptide
PBMC	Peripheral Blood Mononuclear Cells
PCI	Percutaneous coronary intervention
PI	Principal Investigator
PVD	Peripheral vascular disease
RHI	Reactive Hyperaemic Index
ROS	Reactive oxygen species
RSI	Repetitive strain injury

SAE	Serious adverse event
SAP	Statistical Analysis Plan
SASP	Senescence-associated secretory phenotype
SST	Serum Separation Tube
STEMI	ST segment elevation myocardial infarction
SUSAR	Suspected unexpected serious adverse reaction
TA	Telomerase activity
TIA	Transient ischaemic attack
TL	Telomere length
TMF	Trial Master file
TMG	Trial Management Group
TSC	Trial Steering Committee
TTE	Transthoracic echocardiography

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Chapter One
Introduction

1.1 Background

Elderly patients are at high risk of recurrent cardiovascular events after acute coronary syndromes (ACS). While contemporary risk reduction therapies focus on potent antiplatelets and lipid modificatifon, reducing inflammation remains an unmet need. Targeting innate immune system pathways, in particular the interleukin 1B (IL-1B) interleukin 6 (IL-6) axis, decreased adverse cardiac events in post ACS patients, but resulted in increased side effects, including significant infections [1]. Reversing immunosenescence, the decline in adaptive immune system function, is a potential therapeutic target [2]. Senescence of certain T cell subtypes can contribute to atherothrombosis through increased inflammation and reduced immune surveillance. Ageing and cytomegalovirus (CMV) infection are associated with an inflated $CD8^+$ T cell compartment as well as reduction in naïve T cells and T cell receptor diversity [3][4]. ACS can itself trigger immune ageing, reflected by an increase in CD8 terminally differentiated T lymphocytes ($CD8^+ T_{EMRA}$) which are highly pro-inflammatory cells with shortened telomeres. Lymphopenia and a high proportion of $CD8^+ T_{EMRA}$ are negative predictors of survival in unselected octogenarians and in patients following ACS [5][6]. Reducing the proportion of senescent T cells and improving immunity may be achieved by activation of telomerase. The catalytic component telomerase reverse transcriptase (TERT) uses the RNA component (TERC) to synthetize new telomeric deoxyribonucleic acid (DNA). Activation of telomerase has been shown to reverse tissue degeneration in aged mice with severely shortened telomeres[7]. Furthermore, TERT has been shown to reduce oxidative stress[8] and promote vasodilation in arterioles [9] independent of its ability to elongate telomeres.

The only telomerase activator for humans is cycloastragenol (CAG), a compound derived from the roots of the herb astragalus.TA-65MD is a purified and encapsulated form of CAG with enhanced bioavailability (T.A. Sciences). We have demonstrated that TA-65MD stimulates CD4+ T lymphocyte proliferation and telomerase in a TERT-dependent manner [10]. In this pilot phase 2 randomised controlled trial, we sought to evaluate the safety and efficacy of TA-65MD in post ACS patients over the age of 65.

1.2 Acute Coronary Syndromes

1.2.1 Clinical definitions

Acute coronary syndrome is the acute manifestation of atherosclerosis- a slowly progressive chronic disorder of large and medium-sized arteries[11]. Sudden atheromatous plaque rupture with resulting intraluminal thrombus is the pathological landmark of ACS. Reduction in myocardial blood flow may result in myocardial necrosis.

Patients with ACS usually present with chest pain as the primary symptom, with a smaller proportion reporting dyspnoea. ACS can also present with sequalae such as cardiac arrest, electrical instability, cardiogenic shock due to ongoing ischemia or mechanical complications.

In the clinical setting, acute coronary syndromes are divided into 2 groups based on electrocardiogram (ECG) findings.

1) ST-segment elevation myocardial infarction (STEMI)

Patients present with acute chest pain and persistent (>20 min) ST segment elevation. This indicates acute total or subtotal coronary occlusion and warrants emergent revascularisation by primary percutaneous coronary intervention (PCI).

2) Non-ST segment elevation myocardial infarction (NSTEMI)/ Unstable angina (UA)

Patients present with symptoms of myocardial ischemia and no persistent ST segment elevation. ECG may be normal or include the following changes: transient ST segment elevation, persistent or transient ST segment depression, T wave inversion.

In both settings, cardiomyocyte necrosis is measured by an increase and/or decrease of a cardiac biomarker- typically high sensitivity cardiac troponin T or I, with a least one value above the 99th percentile of the upper reference limit[12].

1.2.2 Epidemiology

The Myocardial Infarction National Audit Project (MINAP) recorded 92233 confirmed cases of ACS in the UK in 2017/2018[13]. The majority (61%) were NSTEMIs and 39% were STEMIs. NSTEMIs were more common in the elderly, with a median age of 71 years old. Risk factors for ACS include smoking, diabetes mellitus, hypertension, hypercholesterolaemia and family history of premature coronary artery disease (CAD). There is an increasing prevalence of diabetes in association with increasing age and obesity.

1.2.3 Contemporary management

The acute management of ACS is dominated by initiation of potent antithrombotic drugs and prompt restoration of coronary blood flow by PCI.

Following diagnosis of ACS, patients are initiated on aspirin and a P2Y12 inhibitor. More potent antiplatelet inhibition with prasugrel or ticagrelor are associated with incremental benefit compared to clopidogrel in terms of reduction of ischemic events[14][15]. A dual antiplatelet strategy is maintained for 12 months unless there is an excessive risk of bleeding[12]. Glycoprotein IIb/IIIa inhibitors such as tirofiban can be administered as an adjunct to PCI in the setting of thrombosis or no reflow.

In addition to anti-platelet treatment, anticoagulation is recommended at the time of diagnosis and during revascularisation. In the setting of NSTEMI, Fondaparinux, a factor Xa inhibitor, is administered subcutaneously as part of medical management or during the waiting period for coronary angiography[16]. Unfractionated heparin is recommended intravenously in patients undergoing PCI.

Reperfusion therapy with primary PCI, if achievable in a timely manner, confers a mortality benefit over fibrinolysis in patients with acute STEMI[17]. Current guidelines recommend a maximum delay of 120 minutes from diagnosis to wire crossing occlusion to choose PCI over fibrinolysis. Health care systems have endeavoured to reduce call-to-reperfusion time to maximise the benefit of primary PCI [18]. As such for STEMI patients, quality of care metrics include call to door and door to balloon times.

The optimal timing of an invasive strategy for NSTEMI/UA is based on patient risk stratification[19]. Very high risk patients with ongoing chest pain and dynamic ischemic changes on ECG, cardiogenic shock or malignant arrhythmias should undergo immediate coronary angiography and follow on PCI if appropriate. Patients with stabilised NSTEMI considered at high risk of adverse events (predicted 6-month mortality > 3%) should be considered for a routine invasive strategy within 72 hours. Coronary angiography provides confirmation of the diagnosis, identification of the culprit lesion and overall disease complexity. The decision whether to revascularise with PCI or coronary artery bypass graft (CABG) is based on patient-related and anatomical factors.

Secondary prevention therapies are largely focussed on the aggressive control of low-density lipoproteins cholesterol (LDL-C). PCSK9-inhibition added to intensive statin therapy can produce further incremental gains in outcomes[20]. Finally, cardiac rehabilitation is an effective measure to improve outcomes after ACS[12].

1.2.4 Cardiovascular risk in post-ACS patients

Despite the availability of contemporary treatments, patients remain at a high risk for recurrent ischemic cardiovascular events after ACS. In a large all-comers cohort study of 97254 patients, composite endpoint of cardiovascular death, myocardial infarction (MI), stroke was 18 % during the first year post-index MI [21]. Age was the strongest predictor of ischemic events or death one year after index MI [60–69 vs. <60 years: HR (95% CI): 1.37 (1.30–1.45); 70–79 vs. <60 years: 2.13 (2.03–2.24); >80 vs. <60 years: 3.96 (3.78–4.15)]. Other predictors were prior MI, stroke, diabetes mellitus, heart failure and no index MI revascularisation. One in 5 patients without an endpoint in the first year had an event in the following 36 months.

Despite improvement in reperfusion therapy, post infarct heart failure places a heavy burden on healthcare. The reduction in 5-year mortality following ACS has mirrored an increase in the incidence of heart failure [22]. Over 70% of elderly patients (>65 years old) develop new onset HF within 5 years of a first presentation of MI [23]. Addressing the causes of heart failure post ACS is therefore a priority. The development of 24-hour emergency PCI networks has helped in reducing delays to reperfusion. Symptom to reperfusion time is indeed a key predictor of infarct size. Despite restoration of coronary epicardial flow, microvascular obstruction (MVO) is frequently detected on cardiac MRI. MVO is caused by influx of leucocytes into the infarct zone leading to microvascular plugging, oedema and increased microvascular resistance. This phenomenon leads to myocardial ischemia reperfusion injury and is linked to worse clinical outcomes, negative left ventricular remodelling and larger infarct size[24]. Myocardial ischemia reperfusion injury (IRI) has multiple causes, including metabolic factors, inflammation and microvascular obstruction[25]. However, the clinical significance of IRI is not proven in humans and therapeutic trials targeting different mediators of IRI have not yielded positive results.

1.3 Role of inflammation in Acute Coronary Syndromes

1.3.1 Plaque formation, progression and rupture

Atherothrombosis is the pathological landmark of ACS. Progression of the atherosclerotic plaque is driven by lipid accumulation and complex inflammatory processes in the arterial wall [11].

In the initial stage of atherosclerosis, subendothelial entry of apolipoprotein B-containing lipoproteins triggers the expression of adhesion molecules on the endothelial surface. This leads to the adherence, migration and accumulation of leucocytes. Moreover, vascular endothelial cells secrete monocyte chemotactic protein-1 (MCP-1) and macrophage colony stimulating factor which promote monocyte chemotaxis, adhesion and differentiation into macrophages. T lymphocytes are attracted to the intima by CXC chemokines expressed by endothelial cells, smooth muscle cells and macrophages [26].

Lipoprotein particles bound to proteoglycan in the intima become oxidised and are engulfed by macrophages forming foam cells. Oxidative enzymes such as myeloperoxidase are involved in the production of oxidised LDL. This leads to appearance of early plaques called fatty streaks. Accumulation of inflammatory cells and extracellular lipids lead to maturation of the plaque core which becomes surrounded by a cap of smooth muscle cells and collagen. Secretion of degrading proteases and cytokines by pro-inflammatory immune cells causes the fibrous cap to become vulnerable. Rupture of the plaque leads to discharge of plaque debris into the lumen, driving the release of tissue factor into blood. This in turn triggers the coagulation cascade and thrombosis. The subsequent reduction in blood flow to a region of myocardium triggers infarction.

1.3.2 Post infarct myocardial injury and repair

Following atherothrombosis, potent inflammatory pathways are initiated in the injury and repair of the infarcted heart [27]. The function of the initial phase of inflammation is to clear the infarct from dead cells and matrix debris. Necrotic cardiac cells and damaged extracellular matrix release danger signals which activate the completement pathway and pattern recognition receptors on resident innate immune cells. This triggers a release of pro-inflammatory cytokines, recruiting neutrophils, monocytes and lymphocytes to the infarct site. Release of reactive oxygen species (ROS) from ischemic tissue activates inflammatory signals in the infarcted heart. ROS triggers leukocyte infiltration and induces chemokine and cytokine expression. Activated endothelial cells express adhesion molecules such as selectins and Intercellular Adhesion Molecule 1 (ICAM-1) that facilitate adhesion and transmigration of leucocytes into the infarct zone [28]. The initial post infarction inflammation, which peaks around day 3, can augment ischemic myocardial injury following reperfusion.

The subsequent repair and healing phase last for around 10 days. Scar tissue is deposited at the infarcted region to prevent myocardial dilatation and rupture. The process is characterised by anti-inflammatory signalling, fibroblast proliferation, and deposition of granulation tissue [29].

1.3.3 Role of innate immunity

1.3.3.1 Pattern recognition receptors

The innate immune system provides a rapid and non-specific response to 'danger signals' via specific receptors called pattern recognition receptors (PRRs) [30]. These include Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs).

The PRRs recognise exogenous pathogen associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs) such as proteins, cytokines and chemokines. Interaction of PRRs with their ligands induces intracellular signalling cascade which drives the production of inflammatory cytokines, microbicidal substances and other defensive mediators. While PRR signalling is critical for host defence against pathogen infection, aberrant activation is related to the development of cancer, autoimmune and chronic inflammatory diseases.

TLRs, in particular TLR4 and TLR2, are upregulated in atheromatous plaques [31]. Interaction of oxidised low-density lipoprotein with TLR is a key step in macrophage differentiation to foam cells [32]. In the setting of ACS, there was overexpression of TLR4 on macrophages and TLR2 on granulocytes in thrombi aspirated from patients with ACS[33]. In the infarcted heart, endogenous DAMPS such as heat shock proteins released from dying cardiac cells also signal through TLRs [34].

NLRs are cytosolic sensors of intracellular DAMPS and PAMPs. The NLR-P subfamily is associated with the inflammasome- a macroprotein complex that mediate the inflammatory response upon ligand signalling by converting pro-caspase-1 to caspase-1 [35]. Caspase-1 can trigger a powerful inflammatory reaction by further activating pro-inflammatory cytokines such as IL-1B and IL-18. Experimental data demonstrated NLRP3 inflammasomes are activated by cholesterol crystals in atherogenesis [36].

1.3.3.2 Innate immune cells

Amongst innate leucocytes, monocytes, macrophages and neutrophils have the largest evidence base supporting their role in atheroma progression, plaque rupture and post ACS myocardial inflammation. Macrophages are the most prevalent leucocytes in atherosclerotic plaques and differentiate from monocytes recruited from circulating blood [37]. Macrophages also proliferate locally in advanced plaques. As described in section 1.2.1, macrophages are involved in all phases of atherosclerosis. Based on the expression of CD14 and CD16 on the cell surface, human macrophages are categorised into 3 subgroups[38]. The most common macrophage subgroup is $CD14^{++}CD16^{-}$, known as classical macrophages (85%) which express high levels of C-C chemokine receptor type 2 (CCR2). The intermediate macrophages ($CD14^{++}CD16^{+}$) play a crucial role in atherosclerosis. Non-classical macrophages ($CD14^{+}CD16^{++}$) perform an in vivo patrolling role.

In patients with STEMI treated with PCI, peak levels of classical monocytes, but not non-classical monocytes, were significantly inversely associated with the extent of myocardial salvage occurring 7 days after MI[39]. The mechanism by which $CD14^{+}CD16^{-}$ monocytes increase myocardial damage is unclear. Monocyte chemoattractant protein (MCP)-1, a ligand for CCR2, is significantly upregulated in the ischemic myocardium and it drives recruitment of mononuclear cells into the injured myocardium [40]. The proportion of intermediate monocytes is increased in patients with CAD and is associated with vulnerable plaque morphology [41] [42]and adverse clinical events[43].

Activated macrophages in atheroma release proteolytic enzymes which degrade collagen, leading to thinning and fragilization of the fibrous cap [44]. High macrophage content, in addition to a large lipid pool and thin fibrous cap are considered the key characteristics of the vulnerable atheromatous plaque [45]. Macrophages also produce tissue factor, a major procoagulant, thus linking arterial inflammation and thrombosis [46].

In the initial post ACS phase, neutrophils are recruited to the infarcted heart through a multistep adhesion cascade. Circulating neutrophils are captured by the activated endothelium and interact with the chemokines to become firmly attached to the endothelial surface. After transmigration across the endothelial layer, and extravasation into the infarct, neutrophils have a primary role in the clearance of dead cells and matrix debris [47].

Experimental models have demonstrated a dual role for monocytes and macrophages in the infarcted heart. In the initial phase, release of CCR2, also known as monocyte

chemoattractant protein 1 (MCP-1) drives the infiltration of pro-inflammatory phagocytic monocytes and macrophages into the infarct. Subsequently, reparative subpopulations are recruited to help the healing process. The signals involved in this phenotypic change are unclear.

1.3.4 Role of adaptive immunity

Experiments have demonstrated the contribution of the adaptive immune system, regulated by T cells, to the complex inflammatory response present in all phases of atherosclerosis[48]. Distinct features of T cells were compared in patients with symptomatic and asymptomatic carotid artery disease using single-cell proteomic and transcriptomic analyses. In this immune cell atlas, plaques from symptomatic patients comprised chronically activated and differentiated CD4 and CD8 T cells, with the majority being effector-memory subsets[49]. Histopathological studies reported T-cell infiltrates in both remote and peri-infarction regions as well as within the coronary artery wall of both infarct and non-infarct related arteries in autopsy specimens from patients suffering from MI [50]. In an analysis of atherectomy specimens, patients with ACS had significantly higher proportions of recently activated T cells (interleukin-2 receptor-positive cells) in culprit lesion plaques compared to stable angina patients [51]. Those findings were confirmed in another study assessing a panel of different T cell activation makers (CD25, CD26, CD40L, CD69)[52].Analysis of the T cell population in human plaques revealed a polyclonal population, implying that several antigens are involved in atherogenesis [53]. Antigens proposed to interact with T cells in plaque include oxidised LDL[54], altered matrix components, advanced glycosylation end products, heat shock proteins and micro-organisms [55]. T cell activation particularly in lipid rich plaques can also be mediated by cytokine IL-15 produced by macrophages [56].
The role of T cells in myocardial ischemia reperfusion injury has primarily been demonstrated in experimental mouse models [57][58]. In the acute post ACS phase, effector T cells contribute to the proinflammatory milieu through release of cytokines. They also regulate the infiltration and differentiation of proinflammatory monocytes. Regulatory T cells initiates the healing process and promote an anti-inflammatory milieu by modulating the differentiation macrophages towards a reparative phenotype [59].

1.3.5 Anti-inflammatory therapy

The CANTOS trial validated the inflammatory hypothesis of atherosclerotic disease. It randomised 10,061 patients with previous MI and a high-sensitivity C-reactive protein level (hsCRP) ≥ 2 mg/L to canakinumab, a monoclonal antibody inhibiting the interleukin-1B innate immunity pathway, or placebo. Patients receiving canakinumab at a dose of 150 mg had a significantly reduced rate of recurrent cardiovascular events than placebo, independent of lipid-level lowering. Patients with the largest reductions in levels of interleukin-6 and hsCRP derived the greatest magnitude of risk reduction[1]. However, there was a higher incidence of fatal infection with canakinumab.

Subsequently, the COLCOT trial investigated colchicine to reduce adverse events following MI. Colchicine exerts its anti-inflammatory actions through inhibition of the NLRP3 inflammasome which is responsible for the production of IL-1B and downstream IL-6. 4745 patients were randomised to colchicine 0.5 mg once daily or placebo within 30 days after MI. Colchicine reduced major adverse cardiovascular events (5.5% vs. 7.1%; HR 0.77; 95% CI 0.61-0.96; p=0.02) but was associated with an increased risk of pneumonia (0.9% vs. 0.4%, p=0.03)[60].

The CANTOS and COLCOT trials have demonstrated that inhibiting the IL-1B-IL6 pathway can improve outcomes in patients with established coronary artery disease (CAD). Studies targeting alternative inflammatory pathways have not demonstrated benefit. Administration of low-dose methotrexate in high risk stable CAD patients did not result in a reduction in inflammatory markers or cardiovascular events[61]. Other trials involving the p38 MAP kinase inhibitor (losmapimod) and phospholipase inhibitor (darapladib) were neutral [62] [63]

Targeting different mechanisms of RI have resulted in a reduction in MI size in clinical and experimental models. These include inhibition of mitochondrial permeability transition pore (PTP) opening, metabolic modulation, ischemic postconditioning and remote ischemic conditioning. However, so far, there has been a failure to translate these strategies into reduction of hard clinical endpoints.

1.4 Role of T cells in ACS

1.4.1 T cell subtypes and function

T cells have an immunological memory and are programmed to respond to specific antigens unique for each T cell. Every T cell only recognises one antigen in relation with a major histocompatibility complex (MHC) molecule. CD4 helper T cells recognise antigens in the context of an MHC class 2 molecule expressed by professional antigen presenting cells. CD8 T cells recognise antigens in the context of MHC class 1 molecules expressed by most nucleated cells. Co-receptors on T cells influence T-cell function and fate. Co-signalling molecules include C28, programmed cell death 1 and the tumour necrosis factor (TNF) receptor superfamily including CD40, CD27, OX40 and CD137. A co-stimulatory signal, typically involving CD28 on the T-cell surface, is generally necessary to ensure optimal and sustained activation of the T cells. Moreover, chemokine receptor signalling regulates the migration of T cells from secondary lymphoid organs to sites of inflammation. Once activated, naïve T cells differentiate into central and effector memory T cells. Phenotypically, T cells can be divided into naïve, central memory, effector memory and terminally differentiated effector memory cells. The peripheral T-cell compartment is composed primarily of memory T-cells which, upon antigen recognition, can rapidly proliferate and execute effector function at target sites. T cells secrete cytokines or express surface molecules to modulate the type and intensity of the inflammatory response. They interact with the vascular milieu influencing growth behaviour of vascular smooth muscle cells as well as synthesis and degradation of extra cellular matrix proteins. The T helper type 1 (Th1) response is considered a key driver for atherosclerosis and is characterised by the secretion of cytokines after T cell activation [64]. Interferon-gamma (IFN-γ), the prime Th1 cytokine, is active in the atherosclerotic plaque and it induces several pathogenic effects including upregulation of adhesion molecules, enhanced protease and chemokine secretion, and enhanced activation of macrophages and endothelial cells. CD8 T cells exert their cytotoxic action through the release of the pore-forming protein perforin and other pro-apoptotic proteinases such as granzymes. CD8 T cells also produce pro-inflammatory cytokines such as tumour-necrosis factor (TNF-a) which are cytotoxic in the vicinity of target cells [65].

1.4.2 $CD4^+$ T cells

CD4+ T cells are known as helper cells with effector and regulatory capabilities. They are further subdivided into Th1, Th2, Th17 and regulatory T cell subpopulations.

Experimental models have revealed different contributions of $CD4^+$ T helper cells to atherosclerosis. For instance, Th1 polarised T cells produce the pro-inflammatory cytokine IFN-γ and are considered pro-atherogenic. In contrast, IL-10 producing regulatory T cells limit lesion progression [66]. Naive T-helper cells differentiate into different effector T cells following antigen presentation of the T cell receptor, secretion of cytokines and costimulation. Most naïve $CD4^+$ T cells express the co-stimulatory receptor CD28 which regulates the response of T cells to antigen. T cell activation relies not only on the recognition of antigen, but also a second signal delivered by the interaction of the CD28 costimulatory receptor with its ligand on antigen presenting cells.

$CD4^+CD28^-$ T cells represent a unique subset of T cells that do not express CD28. They share features with classic T helper cells but also diverge phenotypically and functionally from conventional $CD4^+CD28^+$ cells [67]. Unlike the latter, $CD4^+CD28^-$ can function as cytotoxic effector cells. In vitro, perforin-expressing $CD4^+CD28^-$ T cells from UA patients has been shown to exhibit cytotoxic activity against endothelial cells [68]. High levels of perforin and granzyme B were expressed in $CD4^+CD28^-$ in patients with ACS[67]. Another distinguishing feature is the reduced sensitivity of $CD28^-$ T cells to apoptosis [69]. CD28 is necessary to sustain proliferation in classic $CD28^+$ T helper cells. $CD4^+CD28^-$ cells retain their ability to expand and function through the expression of alternative costimulatory receptors, namely OX40 and 4-1BB [67]. In ACS patients, those were significantly upregulated in $CD4^+CD28^-$ compared to classical $CD4^+CD28^+$ cells. $CD4^+CD28^-$ T cells have been shown to recognise heat-shock protein 60 antigens which are expressed by endothelial cells in the setting of ACS [70]. Similar sequences of T cell receptor (TCR) β-chains were isolated from clones of multiple UA patients, suggesting that those clones are expanded in response to common exogenous antigen(s) [71]. The main effector cytokine of $CD4^+CD28^-$ cells is IFN-γ which activates plaque-infiltrating macrophages [67]. A study by Liuzzo et al showed that monocytes from UA patients exhibited a molecular fingerprint of recent IFN-γ triggering, such as nuclear translocation of STAT-1 complexes and upregulation of IFN-γ -inducible genes[72]. $CD4^+CD28^-$ T cells have functional properties that favour plaque instability. Patients with unstable angina had a significantly higher frequency of CD4+ CD28- in peripheral blood compared to stable angina [67], [73]. Patients with NSTEMI and ruptured fibrous cap as culprit lesion on Optical Coherence tomography evaluation had higher levels of peripheral $CD4^+CD28^-$ and $CD4^+CD28^-$ Treg ratio compared to NSTEMIs with intact fibrous cap and stable angina patients. Those

studies together implicate $CD4^+CD28^-$ as a biomarker of plaque instability characterised by fibrous cap thinning and rupture[74]. It has been shown that the expansion of those pro-inflammatory T cells is not a transient phenomenon and rather it persisted for at least 3 months after ACS[75]. Patients with recurrent ACS after the index episode were found to have significantly higher frequencies of circulating senescent T cells compared to those with no recurrent events [76] [77]. $CD4^+CD28^-$ T cell frequency measured from peripheral blood samples was an independent predictor of future ACS events. While those studies were limited by small sample sizes, the association between $CD4^+CD28^-$ and outcomes highlights $CD4^+CD28^-$ as a potential treatment target. The same group previously demonstrated that statins and infliximab, an anti-TNF-a monoclonal antibody, reduce $CD4^+CD28^-$ cell frequency in unstable angina patients. Whether this translates into improvement in clinical outcomes is unknown[78], [79]. However, studies investigating the role of $CD4^+CD28^-$ T cells in CVD prediction have generated conflicting results. A nested case-control study within MESA and CHS showed no statistically significant associations of $CD4^+CD28^-$ memory with first incidence of MI or angina[80]. Interestingly, in a population-based case-control study within Malmo Diet and Cancer study, higher $CD4^+CD28^-$ levels were associated with a lower incidence of first time coronary event during a follow up period of up to 17 years. The opposite association was found in patients with established atherosclerotic disease [81]. Those studies suggest that T cell phenotypes have limited value as biomarkers for risk prediction in patients free of CHD. In those with established disease, it is not possible to define whether the observed variation in T cell subsets occurred before or after disease onset[82].

1.4.3 $CD8^+$ T cells

$CD8^+$ T cells are typically cytotoxic T cells that kill cells presenting extrinsic and intrinsic antigens (e.g. tumour cells) on MHC class I molecules. The mode of target cell death is usually apoptosis through $CD8^+$ T cell mediated granule exocytosis and delivery of effector molecules such as perforin. Release of cytokines namely TNF-a and INF-Y create a cytotoxic milieu when secreted in the vicinity of target cells.

In atherosclerosis, $CD8^+$ T cells are activated and expand locally in a potentially antigen-dependent manner[83]. Production of cytokines and cytotoxic mediators promote plaque inflammation and cytotoxic cell death of macrophages, smooth muscle

cells and endothelial cells, thus promoting plaque instability[83]. Advanced human atherosclerotic plaques harbour predominantly $CD8^+$ T cells[84] in contrast to the CD4:CD8 ratio of approximately 2:1 normally seen in blood[85]. Experimental studies highlight the role of $CD8^+$ T cells in atherosclerosis progression. Transfer of CD8+ T cells into lymphocyte-deplete Apolipoprotein E-deficient mice (prone to atherosclerosis) was associated with CD8+ T cell infiltration in lesions as well as increased lipid and macrophage accumulation, apoptotic cells, necrotic cores and interleukin 1B in atherosclerotic lesions[86]. Other mouse models have suggested a role for $CD8^+$ T cells in promoting macrophage accumulation in atherosclerotic plaques[87]. In a study using human arterial tissue microarrays to assess the migration of T cells into the intima, $CD8^+$ T cells comprised up to 50% of all lymphocytes in advanced atherosclerotic plaques. Resting cytotoxic cells were able to migrate into the arterial wall when affected by advanced lesions but not at the earliest stages of the disease. However, following T cell receptor and/or pro-inflammatory cytokine activation, T cells migrated efficiently into the arterial intima in healthy or mildly affected sites[88].

Clinical studies have demonstrated higher proportions of cytotoxic $CD8^+$ T cells in patients with coronary artery disease compared to healthy controls. Patients with ACS and stable angina had higher blood levels of IFN-γ producing $CD8^+$ T cells expressing the natural killer cell marker CD56 compared to controls[89]. This difference persisted after 3 and 12 months. In 700 subjects from the cardiovascular arm of the Malmo Diet and Cancer Study, subjects with a high proportion of $CD8^+$ T cells were associated with increased incidence of coronary events [90]. The OPTICO-ACS study produced further insight into CD8 T cells role in acute culprit lesions. Intact fibrous cap lesions compromised higher levels of CD4 and CD8 T lymphocytes, along with effector molecules including granzyme A, perforin and granulysin, as compared to ruptured fibrous cap lesions. In an ex-vivo model, under condition of disturbed laminar flow, endothelial cells demonstrated increased adhesion of CD8 T cells which induced cytotoxic endothelial cell death[91]. $CD8^+$ T cell-induced endothelial damage resulting in plaque erosion may represent a potential cause of ACS. Accumulation of effector $CD8^+$ T cells represent a marker of immune cell ageing and will be discussed in section 1.5.

1.5 Inflamma-aging

1.5.1 Inflammation, ageing and cardiovascular disease

Ageing can be described as a time-related decline in physiological functions. It comprises complex intertwined biological processes that lead to frailty and disease[90]. Among them, inflammation is recognised as a key driver of cardiovascular and cerebrovascular disease, the leading causes of mortality and morbidity worldwide. The term 'inflamma-ageing' describes a state of chronic, low grade, systemic inflammation that develops with age in the absence of infection[92].

Lope-Otin enumerates 9 hallmarks of ageing: (1) genomic instability, (2) shortening telomere length, (3) epigenetic modifications, (4) loss of proteostasis, (5) deregulated nutrient sensing, (6) mitochondrial dysfunction, (7) cellular senescence, (8) stem cell exhaustion and (9) altered intracellular communication, all associated with sustained inflammation[93].

Inflamma-ageing is characterised by dysfunctional immune cells and molecules associated with both inappropriate pro-inflammatory response and inadequate defence against foreign pathogens[94]. Physical, chemical and metabolic stimuli perpetuate a chronic inflammatory state. However, there is a lack of standard measures to characterise chronic inflammation. The utility of current biomarkers of inflammation as a surrogate of inflamma-ageing is not clear. Pro-inflammatory cytokines such as IL-1 and TNF-a demonstrate inconsistent trends with age[95]. Sayed et al performed machine learning on blood immune biomarkers to predict multiple aging phenotypes and all-cause mortality in different cohorts[96]. The strongest contributor was the chemokine CXCL9 involved in cardiac ageing, adverse cardiac remodelling, and impaired vascular function. Silencing CXCL9 reversed loss of function, senescence, and hallmarks of arterial stiffness in ageing endothelial cells in humans and mice.

1.5.2 Cellular senescence in atherosclerosis

Cellular senescence is the irreversible loss of proliferation ability of cells over time. It is characterised by critically shortened telomeres, DNA damage/ defective DNA repair and oxidative stress [97]. While ageing is the main driver of cellular senescence, inherited defects in DNA repair enzymes or lamin as well as cardiovascular risk factors such as smoking and diabetes can accelerate this process. Senescent cells undergo widespread change in protein expression and secretion, including pro-inflammatory

cytokines, chemokines and proteases, termed the senescence-associated secretory phenotype (SASP) [98]. SASP also triggers increased production of reactive oxygen species [99].

Senescent cells influence surrounding cells via SASP and contribute to age-related conditions namely arterial stiffness, atherosclerosis and myocardial remodelling [100]. Cardiomyocytes are post-mitotic cells that demonstrate senescence through telomere shortening independent mechanisms such as DNA foci damage and mitochondrial dysfunction. Cardiomyocytes may develop an atypical SASP and express profibrotic and hypertrophic mediators. In aged mice, treatment with a senolytic drug (navitoclax) eliminated senescent cardiomyocytes and reduced profibrotic protein expression. This was associated with improved myocardial remodelling and diastolic function following MI [101].

There is mounting evidence showing the key role of cellular senescence in development and progression of the atherosclerotic plaque. Endothelial cell senescence with loss of function [102] and a shift towards proinflammatory phenotype enhances migration of inflammatory cells. Reduced proliferation and increased apoptosis of vascular smooth muscle cells compromises the integrity of the atherosclerotic plaque.

1.5.3 T cell immunosenescence

Immunosenescence is the decline of the immune system, primarily involving changes in the T cell compartment (**Figure 1**). In vitro, T cells are unable to divide and are susceptible to activation-induced apoptosis following antigen-specific stimulation [103].

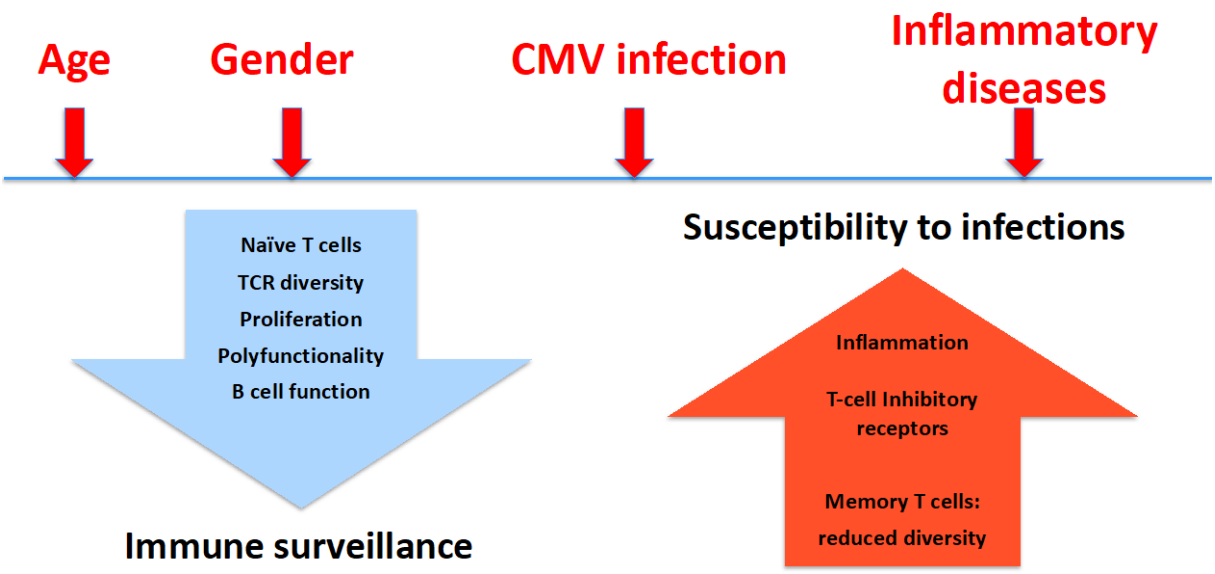

Figure 1. Immunosenescence – risk factors, reduction in immune surveillance and increased susceptibility to infection

A well-functioning innate system requires the generation of a diverse and well-balanced T cell repertoire. Ageing is associated with an overall reduction in the naïve T cell repertoire, partly explained by thymus involution [104]. The $CD8^+$ naïve T cell compartment is the most affected. It is noted that the level of naïve $CD4^+$ T cells stays relatively stable up to the 8^{th} decade of life while the $CD8^+$ naïve T cell compartment suffers gradual sustained loss from mid adulthood. The Swedish OCTO and NONA Immune longitudinal studies described the changes in the innate immune system of the very elderly. In particular, a reversal of the ratio of $CD4^+/CD8^+$ was associated with increased mortality. This 'immune risk phenotype' was driven by an increase in senescent $CD8^+$ T cells [105] [3]. Polychromatic flow cytometry has enabled more detailed analyses of T cell subsets in ageing. $CD4^+$ and $CD8^+$ T cells can be divided on the basis of their expression of the leukocyte common antigen isoform CD45RA and the chemokine receptor CCR7 into naïve (N; $CD45RA^+$ $CCR7^+$), "central" memory (CM; CD $45RA^-$ $CCR7^+$), "effector" memory (EM; $CD45RA^-$ $CCR7^-$) and "terminally differentiated" effector memory (TEMRA; $CD45RA^+$ $CCR7^-$) cells [106][107]Within each of these subsets, the expression of the major T cell costimulatory receptors CD27, belonging to the TNF receptor family, and CD28, belonging to the B7 receptor family can help identify more ($CD27^-$ $CD28^-$) or less ($CD27^+CD28^+$, $CD27^-CD28^+$ or $CD27^+CD28^-$) differentiated cells.

In a study evaluating age-related differences in T cell subsets based on the aforementioned cell surface receptors, the elderly had increased $CD8^+$ T_{EMRA} and decreased CD8 naïve cells compared to the young. There were similar but less marked trends in CD4 cells [108]. Immunosenescent CD8+ cells tend to lose the CD28 coreceptor before losing the CD27 coreceptor[107]. Inflation of $CD28^-CD8^+$ T cells is considered a key component of immunosenescence [109]. Following several rounds of activation with persistent antigenic stimulation, CD28 expression is progressively and irreversibly downregulated resulting in a persistent clonal expansion of highly differentiated $CD28^-$ $CD8^+$ T cells [109]. The latter undergo systematic change in protein expression and secretion, including pro-inflammatory cytokines, chemokines and proteases, termed the senescence-associated secretory phenotype. Moreover, these cells have a reduced proliferative capacity, shortened telomeres and enhanced cytotoxic activity. Expansion of these cells is at the detriment of TCR diversity, reducing overall immunity.

1.5.4 Role of CMV in T cell immunosenescence

Latent infection with human CMV is considered a key driver of age-related $CD8^+$ T cell expansion and senescence. Highly differentiated CD4 and CD8 T cells that re-express CD45 RA increase during ageing and after persistent CMV infection. They are characterised by reduced proliferative capacity, increased activation of senescence signalling pathways and susceptibility to apoptosis(47). It is thought that chronic CMV infection may contribute to atherosclerosis development by promoting a pro-inflammatory environment within the vascular bed. Examination of arterial specimens using PCR showed that a much higher percentage (90%) of patients with atherosclerosis contained CMV DNA in their arterial walls as compared to controls [110]. ApoE knockout mice infected with CMV had significantly increased atherosclerotic burden compared to controls, suggesting a causal relationship of CMV to atherogenesis [111].

Experimental studies have suggested that CMV is delivered to the arterial wall by infected monocytes. CMV Immediate Early gene expression is enhanced in the vascular milieu, thus contributing to viral reactivation and replication [112]. Infection of endothelial and smooth muscle cells initiates series of pro-atherogenesis effects including smooth muscle cell proliferation[113], expression of cytokines and chemokines and increased procoagulant activity of endothelial cells [114]. It is

proposed that some infectious pathogens including CMV can produce systemic alterations that predispose to atherosclerosis without the need to reside in the vasculature. Molecular mimicry resulting in inappropriate immune responses to self-proteins is hypothesized to be the underlying mechanism [115]In a study of rats infected with CMV, increased neointimal response to injury was noted despite absence of the virus from affected segments of the vessel.

The T-cell compartment exhibits the most CMV-related phenotypic alterations. CMV seropositivity in the elderly is associated with clonal expansion of CMV-specific $CD8^+$ T cells with membrane surface marker expression indicative of effector memory cells ($CD28^-$, $CD57^+$, $CCR7^-$) [116]. These cells have low proliferation potential and are considered senescent [117].

The Newcastle 85+ study was the first to investigate the impact of CMV seropositivity and individual immune cell parameters on long term survival in octogenarians.

CMV seropositivity was associated with the immune risk phenotype (lower CD4/CD8 ratio) driven by an increase in $CD8^+$ T_{EMRA} cells. Notably there was a large increase in $CD27^-$ $CD28^-$ subpopulations across all CD4 and CD8 memory T cells in CMV seropositive patients [6]. The latter were more likely to have CAD compared to CMV seronegative participants. CMV seropositivity was linked to increased six-year cardiovascular mortality. Low percentages of senescent CD4 and CD8 T cells reduced the risk of cardiovascular death. Likewise, a high number of $CD27^-CD28^+$ CD8 EMRA T-cells, characterised by retained differentiation plasticity and proliferative capacity [118], protected from both non-cardiovascular death and cardiovascular death.

Epidemiological studies have demonstrated the link between CMV seropositivity and coronary artery disease [119], [120]. In a cohort study of 511 individuals aged at least 65 years old followed up for 18 years, 70% were CMV seropositive. CMV infection was associated with an increased annual mortality rate (Hazard ratio = 1.42, 95% CI: 1.11-1.76), driven by increase in vascular deaths, after adjusting for age, sex and baseline socio-economic and health variables [121]. This translated into shortening of lifespan by approximately 4 years. An analysis of long-lived families in the Leiden Longevity Study demonstrated that offspring from individuals genetically enriched for longevity are less susceptible to the characteristic CMV-associated age-driven immune response. This may signify improved immune control of the virus, leading to a lower mortality rate.

However, the association between CMV seropositivity and CAD has not been consistently proven. First, it is possible that the atherogenic effects of CMV is affected

by the degree of inflammatory response of the host to the infection. One study found that only seropositive CMV individuals with elevated CRP had statistically significant elevated odds ratio for CAD after adjusting for confounders [122]. Similarly, CMV seropositivity was independently associated with increased cardiac death only in the context of IL-6 elevation [123]. Second, testing for CMV seropositivity alone cannot reveal whether there is chronic reactivation of the virus. In one study, active CMV replication in peripheral blood monocytes could be detected in very few healthy adults (2%), but was far more frequently observed in patients with ACS (15%), despite the same prevalence of seropositivity (70-80% in this study) [124].

1.5.5 T cell immunosenescence in ACS

Few clinical studies have assessed the kinetics of senescent T cells in ACS and their potential role in post infarct injury. Hoffman et al described depletion of CD4 T cells from peripheral blood during the first 30 minutes of myocardial reperfusion after primary PCI, driven by a reduction in CD4 CCR7+ T cells. These were typically central memory T cells. It is not clear whether this result implies a CCR7-mediated redistribution of this T-cell subset to peripheral lymphatic organs or a specific accumulation in the re-perfused myocardium. Of note, this study did not specifically evaluate CD8 T cell subsets. The same group then investigated whether AMI activates an inflammatory T-cell response that might provoke accelerated immunosenescence in CMV-seropositive patients.

In a longitudinal analysis of 34 patients undergoing primary PCI, CD8 T cells, in particular CD8 effector memory TEM and TEMRA, were significantly reduced in CMV seropositive patients 30 minutes after reperfusion compared to CMV seronegative patients. Absolute cell counts of CD8+ TEM, but not TEMRA, had completely recovered 24 hours after infarction. The loss of senescent $CD8^+$ T_{EMRA} was pronounced in younger patients (<= 55) but not in older patients (>55). There was overall a persistent depletion of TEMRA cells and cytomegalovirus-specific cytotoxic T cells at 3 months following AMI. It was noted that peripheral blood mononuclear cells (PBMCs) from CMV seropositive patients displayed higher T helper type 1 response than seronegative patients prior to primary PCI determined by IFN-γ enzyme-linked-immunospot assay.

A separate cross-sectional study of 54 patients confirmed a lower percentage of $CD8^+$ T cells 24 hours post STEMI compared with healthy controls or patients with stable

CAD. The loss of virus-specific CD8+ T lymphocytes has been associated with PD-1 (programmed death-1) which is increased on the surface of memory T cells upon activation. After several months post MI, the TEMRA pool increased in CMV seropositive but demonstrated signs of terminal differentiation, including shorter telomere lengths.

The fall in T cell lymphocytes in the context of PCI was further explored in a prospective observational study of patients presenting within 6 hours of STEMI. Lymphocyte count fell acutely after reperfusion, primarily due to loss of T cells. CD8+ T cells decreased more than CD4 with the largest decline in effector subsets from the peripheral circulation (-66% for $CD8^+$ T_{EMRA}). This was associated with the development of MVO- a marker of myocardial IRI. Importantly, T cells were likely sequestered within the myocardium as suggested by measurement of transcoronary gradients.

The role of fractalkine in the reduction in effector T cells was investigated. This chemokine and its receptor CX3CR1 are key to the migration and adhesion of effector T cells and natural killer cells to the vascular endothelium. It exists both as a membrane-bound adhesion molecule and a soluble secreted form acting as a conventional chemoattractant. The CX3CR1 receptor was the prominent chemokine receptor expressed on effector T cells, showing a strong correlation between level of expression in T cell effector subsets and their depletion following reperfusion. The acute decrease in CX3CR1 expression in effector subsets-likely due to ligand binding-suggest a role for fractalkine-mediated margination of T cells to the vascular endothelium within the reperfused microcirculation. Yu et al characterised the functional properties of senescent $CD57^+$ $CD8^+$ T cells in 58 patients presenting with acute MI [125]. CD57 is a surrogate marker of replicative senescence of T cells. In this study, the $CD57^+$ cell population overlapped considerably with the CD28- cell population. $CD57^+$ CD8 T cells expressed higher levels of tissue homing markers notably CX3CR1, secreted higher amount of IFN-γ or TNF-a and greater cytotoxic function than the CD57- $CD8^+$ population. Interestingly, the frequency of $CD57^+$ $CD8^+$ T cells was independently associated with 6-month cardiovascular mortality.

1.5.6 Impact of telomere length shortening on immunosenescence

T cell immunosenescence has been linked to telomere disruption. Telomeres are dynamic complexes of repeat DNA sequences and associated proteins that protect the ends of chromosomal DNA[126]. They are widely regarded as the internal biological

clock of a living organism and have a key role in maintaining cellular function[127]. Telomeres shorten by a few base pairs, i.e., lose part of the DNA sequence with every cell division.

When a critical telomere length is reached, cellular senescence occurs with alterations in morphology and secretary phenotype. Senescent cells promote inflammation by secreting IL-6, IL-8, intercellular adhesion molecule 1 (ICAM-1), metalloproteases and monocyte attractants. Chronic inflammation creates a vicious circle which amplifies telomere dysfunction and increases cellular senescence, leading to age-related chronic illnesses[128]. Independent of traditional vascular risk factors, shorter leukocyte telomeres are linked to the development of CAD as well as higher cardiovascular risk and death. [129]–[132]. Offspring with familial risk of CAD had a lower mean leukocyte TL compared to controls, equivalent to about 17 years of age-related attrition in TL, supporting a genetic link and therefore a primary role of TL shortening in CAD [133] . Moreover, in the West of Scotland Primary Prevention Study (WOSCOPS) study, leukocyte TL were compared at recruitment in patients who eventually developed CAD versus matched controls who remained event free. There was a significant correlation between TL and the risk of developing a CAD event. Interestingly, in individuals treated with pravastatin, the increased risk with shorter telomeres was significantly reduced [134]. A large Mendelian randomization study in 290000 participants assessed 3 single nucleotide polymorphisms associated with shorter TL, namely in the genes TERT, TERC and OBFC1. Odds ratios for ischemic heart disease were 1.05 (1.03-1.08) for OBCF1, 1.04 (1.02-1.06) for TERT, and 1.01 (0.99-1.03) for TERC. A genetically determined 200-bp-shorter telomere length was associated with an odds ratio for ischemic heart disease of 1.10 (1.06-1.14), inferring a causal relationship [135]. Using virtual histology intravascular ultrasound studies, Calvert et al proposed that the higher cardiac events associated with low LTL could be related to high-risk ‘inflamed’ plaque subtypes rather than total plaque burden[136].

The association between short LTL and atherosclerosis has not been universally proven. A cross-sectional study showed that average LTL and short telomere load were not independently associated with subclinical atherosclerosis in a middle-aged population [137]. An important limitation of studies evaluating association of TL to atherosclerosis is the use of unselected peripheral mononuclear blood cells which comprise cells that have varying contribution to the disease process. Spyridopoulos and co-workers reported telomere length (TL) across 12 leukocyte subpopulations in CAD patients and healthy individuals[138]. Leukocyte TL in patients with CAD was 500

bp shorter than in age-matched control subjects, a difference that was preserved throughout the haematopoietic system independent of myeloid or lymphoid origin. The exception was a significant TL deficit of 1.0 kb in cytotoxic CD8+ T cells in the presence of CAD. This was particularly marked in CD8+CD28- T cells of CMV seropositive CAD patients compared to seropositive healthy individuals. Interestingly, TL shortening of terminally differentiated CD8+ T cells, which are mostly CMV specific, correlated with degree of LV dysfunction in CAD patients. This study suggests that telomere shortening in leukocyte subpopulations is either inherited or reflects accelerated telomere shortening in common precursors of the subpopulations, caused by systemic factors such as oxidative stress.

Short TL in peripheral blood leukocytes may 1) have an inherited component without the presence of DNA damage, 2) signify higher cell turnover during systemic low grade inflammation, or 3) act as a proxy for accelerated DNA damage and cell senescence.

1.6 Telomerase

1.6.1 Structure and Nuclear function

Telomeres shorten during each cell division cycle since DNA polymerase is unable to replicate the 3' ends of chromosomes fully. At a critical short telomere length, the DNA repair system is activated, inducing replicative arrest, senescence and cell death. Telomere loss is a critical event linked to tissue atrophy, organ dysfunction and stem cell depletion [139][140].

Telomerase is a large ribonucleoprotein complex consisting of the telomerase RNA component (TERC) and TERT. It is responsible for maintenance of telomere length. The catalytic component TERT uses the TERC as a template to synthesize new telomeric DNA repeats (TTAGG) at the 3' ends of linear chromosomes[128]. Telomerase activity has a key role in maintaining cellular proliferation[141].

The relevance of telomerase in maintaining a healthy human lifespan can be inferred from segmental premature ageing (progeroid) syndromes, such as dyskeratosis congenita, which show mutations in TERC, resulting in short telomeres [142]. Mice lacking telomerase exhibit an age-related phenotype and have shorter lifespans [143]. A recent study showed that telomerase activation reversed tissue deterioration in aged

telomerase-deficient mice[144]. This suggests that telomere rejuvenation strategies for age-associated diseases may become a therapeutic target.

1.6.2 Extra-nuclear functions of telomerase

There is evidence that cell physiology is influenced by TERT, regardless of its role in telomere lengthening[145]. In a mouse model, increased TERT, and not TL, was required for the cardiovascular benefits of physical exercise. TERT-deficient animals despite normal TL were not protected against doxorubicin induced cardiotoxicity and vascular apoptosis[146]. Both ageing and coronary artery disease are associated with increased intracellular ROS and a loss of TERT activity[147]. In the setting of oxidative stress, TERT is reversibly excluded from the nucleus in a dose and time dependent manner, leading to premature senescence[148]. Extranuclear telomerase colocalises with mitochondria and in TERT overexpressing cells, mitochondria DNA is protected from oxidative stress with concurrent reduction in cell peroxide levels and mitochondrial superoxide production[8][149]

Using multiple mouse models, Richardson et al showed that oxidative stress resulted in reduced CD4 proliferation, but not monocytes, by suppressing telomerase. Telomerase did not affect the proliferation and function of regulatory T cells if the telomere length was not critically short. However, critically shortened telomeres negated the suppressive action of regulator T cells on effector T cells[10].

Furthermore, reactive oxygen species reduce nitric oxide levels, leading to loss of its vasodilator and anti-inflammatory properties in the microcirculation. Ait-Aissa et al showed that loss of TERT but not TERC contributes to increased microvascular ROS levels and reduced dilatation[150]. Beyer and colleagues have demonstrated that incubating human arterioles with a telomerase inhibitor led to pathological H_2O_2 replacing nitric oxide as the mediator of flow-mediated dilation (FMD). These outcomes signalled by mitochondrial TERT are independent of transcription. On the other hand, telomerase activity shifts FMD back to NO from H2O2[9]. Those studies therefore demonstrated a non-canonical function of telomerase in establishing a physiological mechanism of vasodilation in arterioles and a new target for decreasing oxidative stress in the microcirculation of patients with CAD.

Treatment with pharmacologically active doses of atorvastatin led to a significant increase of telomerase activity in human and mouse PBMC and CD4 T cells, resulting in a moderate proliferation of T lymphocytes. This was negated by the addition of LDL cholesterol and absence of the TERT component of telomerase. Given that it may

protect leukocytes from oxidative stress without impacting the maintenance of their telomere length, this non-canonical function of TERT may be especially significant in understanding the role of telomerase in atherosclerosis[145].

1.6.3 Role of telomerase in atherosclerosis/ cardiovascular system

Telomerase is expressed in low but functionally significant amounts in differentiated low proliferating cells of the cardiovascular system[151]. Mouse models suggested a reduction in telomerase activity with ageing and a marked increase in response to cardiac injury**.** Upregulation of telomerase was detected in cardiomyocytes, endothelial cells, fibroblasts of cryoinjured adult mice hearts[152]. Telomerase activation after MI in adult mice showed reduced cardiac remodelling, improved ventricular function and smaller infarct in TERT-expressing hearts[7]. Hence, those studies suggest telomerase activation may help prevent heart failure after MI.

Compared to age and gender-matched healthy controls, shortened telomeres have been observed in endothelial cells of atherosclerotic lesions, implying loss of telomerase activity in the diseased coronary arteries[153].Moreover, vascular smooth muscle cells in advanced human atherosclerotic lesions are characterised by low levels of TERT[154]. Telomerase expression was able to delay senescence in vascular smooth muscle cells with short telomeres.

There are limited clinical studies measuring leukocyte telomerase activity in atherosclerotic plaques. In a study of 26 patients, Narducci et al evaluated telomerase activity in polymorphonuclear neutrophils (PMN) isolated from peripheral blood and coronary atherosclerotic plaques. Telomerase activity was higher in coronary plaque PMN of UA patients versus SA patients, particularly when PMN was collected within 40 hours of last angina symptom. Peripheral PMN telomerase activity was negligible. These findings suggest local extended activity of the inflammatory cells in the early phase of instability. Another study demonstrated activation of telomerase in macrophages during atherosclerosis development in LDL-receptor-deficient mice. High levels of TERT were also expressed in macrophages of atherosclerotic lesions[155]. We speculate that higher levels of telomerase activity observed in unstable plaques is a marker of increased replicating inflammatory cells in such lesions. Those cells can transiently increase telomerase activity during successive stimulations with accompanying telomere loss[156]. Accordingly, fewer atherosclerotic lesions were detected in telomerase-deficient mice $TERC^{-/-}$ $ApoE^{-/-}$ compared to

$TERC^{+/+}$ $ApoE^{-/-}$ mice [142]. Later generation $TERC^{-/-}$ mice were characterised by spleen and bone marrow defects, thus limiting differentiation and proliferation of immune cells participating in atheroma progression[157].

There is limited data on telomerase regulation in lymphocytes from patients with CAD or acute MI. Following an acute atherosclerotic plaque event, we expect a reduction in telomerase activity due to an increase in terminally differentiated inflammatory cells. In a study of 153 elderly patients (mean age 81) with NSTE-ACS, low TA or short TL in PBMCs were not associated with the incidence of adverse cardiac outcomes – death, MI, unplanned revascularisation, stroke[158].

1.6.4 Telomerase activator

TA-65MD is a purified and encapsulated form of CAG, also named TA-65, with enhanced bioavailability (T.A. Sciences, New York). TA-65 is a mild telomerase activator from an extract of the Astralagus roots. TA-65 MD is marketed in the United States as a nutritional supplement. The compound is manufactured under Good manufacturing practice and has been designated as GRAS (generally recognized as safe) by the FDA for use in medical food up to 100 mg/day.TA-65 MD has been shown to induce telomerase in different tissues in mice and in human lymphocytes and epithelial cells[159], [160]. TA-65MD leads to telomerase-dependent lengthening of short telomeres and recovery of associated DNA damage by increasing TERT-mRNA transcription.

Pharmacokinetics

The telomerase activator TA-65 acts through TERT. TA-65 has been shown to activate telomerase in-vitro. Molgora et al demonstrated that TA-65 increased telomerase activity significantly 1.3 to 3.3 fold relative to controls in human T cell cultures [161]. Pilot experiments conducted in Professor Spyridopoulos' lab tested the effect of TA-65 in human as well as murine lymphocytes. TA-65 up-regulated proliferation in a dose-dependent way (**Figure. 2a and 2b**). TA-65 also induced a dose-dependent up-regulation of telomerase activity over 5 days in *in vitro* culture of activated human PBMCs (**Figure. 2c**). At higher doses, TA-65 does not induce telomerase, and in fact seemed to inhibit it at the maximum concentration of 100μM, a dose far exceeding pharmacological relevance.

To confirm that TA-65 is indeed mediated through TERT, as has been previously suggested through transcriptional activation of TERT mRNA [162], the effect of TA-65 on the growth of splenocytes from $TERT^{+/+}$ and $TERT^{-/-}$ mice was tested [Fig. 2d and e]. Increasing oxidative stress by culturing the cells under hyperoxia was sufficient to abrogate telomerase activity to baseline levels regardless of TA-65 treatment. As expected, hyperoxia was able to suppress baseline T cell growth in $TERT^{+/+}$ lymphocytes; an effect of hyperoxia on $TERT^{-/-}$ was not observed, which suggests that the mechanism of abrogation is mediated through TERT. In summary, the pilot data demonstrate that 1) TA-65 activates telomerase in murine and human lymphocytes, 2) strongly enhances the immune response in these cells, and 3) acts via a TERT-dependent mechanism.

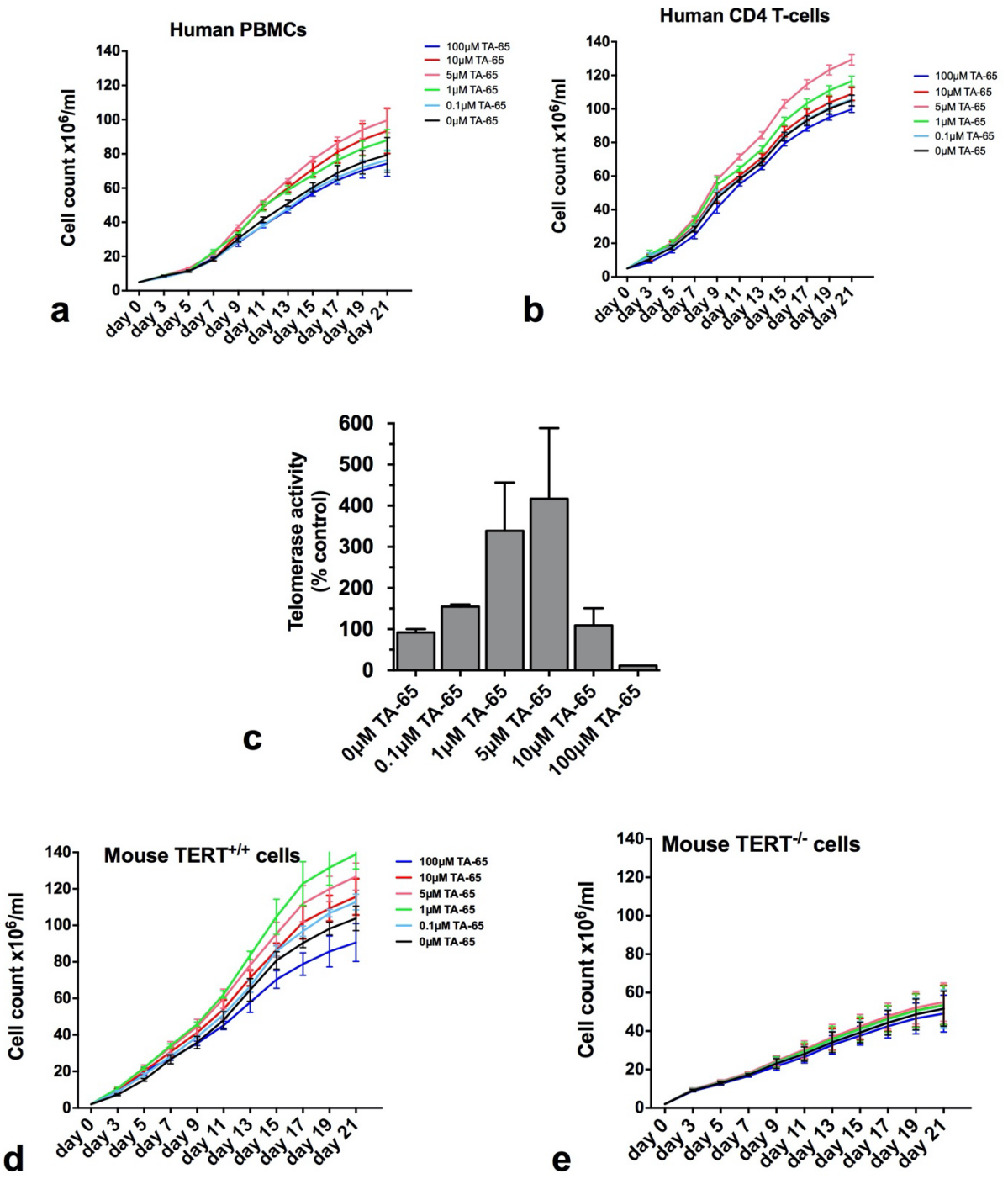

Figure 2. The telomerase activator TA-65 acts through TERT. Human PBMCs (a) and CD4 T-cells (b,c) were activated by adding α-CD3 and α-CD28 specific beads in the absence (black line) or presence of the telomerase activator TA-65. The strongest induction of proliferation was seen at a concentration of 0.5 µM TA, over 21 days (a,b) and telomerase (c) were seen in CD4 T-cells. The effect of TA-65 was also tested in splenocytes from $TERT^{+/+}$ and $TERT^{-/-}$ mice (d,e), documenting the dependency of the TA-65 effect from TERT.

Oral evaluation in humans

Over the course of a one-year observational study of TA-65, a total of 114 adult human volunteers (mean age 63 ± 12 years) participated in a study for varying periods of time (3-12 months) with a starting dose of 5-10 mg/day CAG (unformulated TA-65)[163]. Telomerase in cultured human keratinocytes, fibroblasts and immune cells were moderately increased by low nanomolar levels of TA-65. Among CMV positive participants, a significant reduction in the percent of senescent $CD8^{+}CD28^{-}$ was observed in a linear trend over 12 months. In a subset of patients, there was a significant reduction in the percent short (<4 kbp) telomeres although mean telomere length did not increase. No adverse events (AEs) were reported. Two subjects (out of 114 total participants) self-reported “anxious” feelings soon after voluntarily increasing their daily consumption of TA-65 to 100 mg/day, a consumption level two times the intended dose for this observational study. The feelings disappeared in both subjects when daily consumption was reduced to 50 mg/day. Subsequently in a randomized double blind and placebo-controlled study conducted in Spain, 117 participants were randomized to placebo, 250 units TA-65 MD (8 mg) and 1000 units (32 mg) TA-65[163]. The placebo group had an average telomere attrition of 290 ± 100 bp/year (p = 0.01) while the low dose TA-65MD (250 units) group had net increase of 530 ± 180 bp/year (p = 0.005). Interestingly there were no statistically significant changes in telomere length in the high dose TA-65MD (1000 units) group. The results raise the possibility of a bell-shaped dose response curve. Another explanation is that the high dose TA-65MD (1000 units) led to rescue of near senescent cells resulting in a reduction in the median telomere length. There was no evidence of drug-related toxicities as assessed by biochemical makers of liver, kidney, and metabolic function.

Dose rationale

Based on the animal studies, the no-observed-adverse-effect level (NOAEL) for oral TA-65 was greater than 150 mg/kg/day in male and female rats, equivalent to 10,500 mg/day in a 70-kg individual, which is orders of magnitude higher than the highest doses seen in human pharmacokinetic studies. TA-65MD is currently available as 250 unit capsules (with 8 mg of CAG). Current recommendation for use is 1-4 capsules per day (8-32 mg of CAG/day) which is several orders lower than the NOAEL dose set in the animals.

Human pharmacokinetic data suggests a dose dependent increase in the AUC of CAG with increase in the TA-65MD intake from 1 to 4 capsules (TA Sciences IB). However, dose dependent response of TA-65MD for telomere length could not be accurately ascertained based on existing evidence. In a pilot RCT investigating the effects of TA-65MD on age related macular degeneration, treatment with 500 units TA-65MD (16 mg) resulted in clinical improvement without AEs [164]. Hence, the dose of 500 units TA-65MD has been proposed for future clinical testing.

Chapter Two
Trial rationale and Objectives

2.1 Trial Rationale

Immunosenescence with telomere shortening in peripheral blood leukocytes, increased oxidative stress and inflammation, and impaired microvascular endothelial function accelerate the development of CHD. We hypothesize that activating telomerase with TA-65MD will lead to reduced immunosenescence, decreased telomere shortening, and improved endothelial function in patients with CHD. The null hypothesis is that there will be no difference in immunosenescence between the two groups following 12 months of treatment with TA-65MD or placebo. The choice of active treatment versus placebo is appropriate in this population where no telomerase activator is currently used as part of usual care. All patients will receive usual care alongside the trial[127].

2.2 Objectives

2.2.1 Primary Objective

We aim to assess the effect of 8 mg twice daily of oral TA-65MD given for 12 months on immunosenescence in older patients following ACS.

2.2.2 Secondary Objectives

The secondary objectives of this trial are as follows:

1. To investigate the effect of 1-year TA-65MD treatment on leukocyte telomere length.
2. To investigate the effect of 1-year TA-65MD treatment on microvascular endothelial function.
3. To investigate the effect of 1-year TA-65MD treatment on systemic inflammation and heart failure, reflected by expression of N-terminal fragment of the prohormone brain-type natriuretic peptide (NT-proBNP) and high-sensitivity C-reactive protein (hsCRP).
4. To investigate the effect of 1-year TA-65MD treatment on measures of cardiac function as measured by echocardiography.
5. To investigate the effect of TA-65MD treatment on telomerase activity (TA) and oxidative stress.

6. To investigate the effect of TA-65MD treatment on clinical events—all-cause death, stroke, or MI—in patients after 1 year.
7. To characterize the AE profile of TA-65MD·
8. To quantify adherence to study drugs.
9. To investigate the impact of seropositivity to CMV at baseline on trial outcomes.

Chapter Three
Methods

3.1 Trial Design, Approvals and Setting

The TACTIC trial was a single centre, randomised, double-blind, placebo-controlled phase II trial comparing TA-65MD with placebo in elderly participants following ACS in the 6 months prior to consent. Ninety patients were randomised to either TA-65MD, (n=45), or placebo (n=45), taken twice daily for 12 months.

The trial was conducted according to the International Conference on Harmonisation (ICH)-Good Clinical Practice (GCP). TACTIC was registered at the International Standard Randomized Controlled Trial Number (ISRCTN) registry (16613292) and at the European Union Drug Regulating Authorities Clinical Trials Database (EudraCT), European Union Clinical Trials Register (2017-002876-26). A favourable ethical opinion was granted on August 18th 2018 by the UK Health Research Authority (18/NE/0178). The trial was funded by TA-Science (New York, US) as an investigator-led grant.

All patients were recruited and followed up at a single NHS site (The James Cook University Hospital, Middlesbrough). Blood samples were analysed both at The James Cook University Hospital laboratories, and, where specialist equipment and expertise were required, at the Institute of Genetic Medicine, Newcastle University, International Centre for Life under the supervision of Professor Spyridopoulos.

3.2 Study population

3.2.1 Inclusion criteria

Patients were eligible for the trial if they:

- Provided written informed consent
- Were aged 65 or over with an index presentation of an acute coronary syndrome within the previous 6 months
- Had successfully completed revascularisation or were being managed medically following ACS
- Had angiographic evidence of coronary heart disease (at least one major epicardial vessel stenosis ≥70%)
- Were more than 24 hours after presentation with the index event- patients were eligible the following day after PCI or 3 months after CABG.

3.2.2 Exclusion criteria

Patients were excluded from the trial if they:

- Had any disorder associated with immunological dysfunction (acute or chronic inflammatory or neoplastic co-existing disease, known positive serology for HIV, or hepatitis)
- Were clinically unstable (haemodynamically unstable, cardiogenic shock, unconscious)
- Had severe, uncontrolled hypertension (Blood Pressure >170/110mmHg, or ambulatory BP of 150/95mmHg);
- Had a severe comorbidity that had impact on outcome over next 2 years
- Were taking immunosuppressants
- Had a known malignancy
- Were using a nutritional supplement derived from the roots of the Astragalus species
- Had a previous known substance addiction
- Had insulin-dependent diabetes
- Were judged by the Investigator that they should not participate in the study, for example based on previous serious psychiatric illness or are unlikely to comply with study procedures, restrictions, and requirements
- Had participated in any other interventional medicinal study in the past 6 months

3.3 Outcome measures

3.3.1 Primary outcome

The primary outcome was the proportion of $CD8^+$ T-lymphocytes which were terminally differentiated effector memory cells ($CD8^+$ T_{EMRA}; $CD3^+CD4^-CD8^+CCR7^-CD45RA^+$), measured in peripheral blood with flow cytometry at 12 months. The proportion of terminally differentiated $CD8^+$ effector memory cells (%$CD8^+$ T_{EMRA}) was calculated from the total number of peripheral blood $CD8^+$ T-lymphocytes $CD8^+$. The

mean difference between the intervention and control arms was compared at 12 months. $CD8^+$ T_{EMRA} was chosen as a surrogate for immune ageing, identified among others in the Newcastle 85+ study[6].

3.3.2 Secondary outcomes

Secondary outcomes included the following measurements:

- Proportion and absolute counts of leukocyte subsets (Table 1) at baseline, 6 months and one year.
- Microvascular endothelial function at baseline, 6 months and one year.
- Systemic inflammation (high sensitivity C-reactive protein) at baseline, 6 months and 12 months.
- Cardiac function assessed by both serum levels of NT-proBNP and transthoracic echocardiography at baseline and 12 months.
- Telomerase activity at baseline, 6 months and 12 months.
- Oxidative stress at baseline, 6 months and 12 months.
- Effect of CMV seropositivity at baseline on study outcomes.
- Adverse events

Total Lymphocytes (= $CD3^+$ + $CD19^+$ + NK)	$CD4^+$ N absolute cells (= % CD4 Naïve x CD4 TruCount))	$CD8^+$ N absolute cells (= % CD8 Naïve x CD8 TruCount)
$CD4^+/CD8^+$ ratio (= $CD4^+$ absolute cells / $CD8^+$ absolute cells)	$CD4^+$ CM absolute cells (= % CD4 CM x CD4 TruCount)	$CD8^+$ CM absolute cells (= % CD8 CM x CD8 TruCount)
Total monocytes (= Non-classical + intermediate + classical)	$CD4^+$ EM absolute cells (= % CD4 EM x CD4 TruCount	$CD8^+$ EM absolute cells (= % CD8 EM x CD8 TruCount)
	$CD4^+$ TEMRA absolute cells (= % CD4 TEM x CD4 TruCount)	$CD8^+$ TEM absolute cells (= % CD8 TEMRA x CD8 TruCount)
	$CD4^+$ CD28 negative absolute cells (= % CD4 CD28 negative x CD4 TruCount)	$CD4^+$ CD28 negative absolute cells (= % CD4 CD28 negative x CD4 TruCount)

Table 1. Proportion and absolute counts of leukocyte subsets. N= naïve, CM= central memory, EM= effector memory, TEMRA= Terminally differentiated effector memory, NK= Natural killer cells

3.4 Blood samples

3.4.1 Blood sample collection and handling

Blood samples were taken and labelled by the research team at JCUH. Table 2 provides summaries of the samples collected including time-points, processing and shipping. Blood samples for flow cytometric analysis and telomerase activity were immediately transported to Newcastle Centre for Life at room temperature and processed within 4 hours of being taken. All study blood tubes were labelled using study specific labels and were accompanied by request forms to ensure accountability.

Visit	Sample	Collection tubes	JCUH lab processing	Shipping
○ **Baseline**	CMV	 1x 5 mL SST 454228	Standard virology Practice	n/a
○ **Baseline** ○ **6 months** ○ **12 months**	hsCRP and NT-ProBNP combined sample	 1x 5 mL SST 454228	Standard biochemistry Practice	n/a
○ **Baseline** ○ **6 months** ○ **12 months**	TBARS (Plasma)	 1 x 4 mL EDTA 454021	• Invert 8-10x (In clinic) • Centrifuge for 10 mins at ≤1300 RCF (g) • Aliquot plasma up to 10 x into labelled tubes • Freeze at -80 °C and store until shipment notification	Shipped in batches (every 6 months) to the International Centre for Life To remain frozen in transit
○ **Baseline** ○ **6 months** ○ **12 months**	$CD8^+$ T_{EMRA}	 1 x 4 mL EDTA 454021	• Invert 8-10x (In clinic) • No local lab processing	Shipped immediately to the International Centre for Life at ambient temperature
○ **Baseline** ○ **6 months** ○ **12 months**	Research Bloods	 4 x 9 mL Lithium Heparin 454029	• Invert 8-10x (In clinic) • No local lab processing	Shipped immediately to the International Centre for Life at Ambient temperature
○ **Baseline** ○ **6 months** ○ **12 months**	Future use bloods	 1x 5 mL SST 454228	• Invert 5x, • Allow 30mins to clot • Centrifuge for 10 mins at 1000-Aliquot plasma up to 10 x into labelled tubes • Freeze at -80-°C and store until shipment notification	Shipped in batches (every 6 months) to the International Centre for Life To remain frozen in transit

Table 2. Summary of blood samples collected. SST= serum separating tube, EDTA= Ethylenediaminetetraacetic acid

3.4.2 Flow cytometric assays

Two different assays were used in this study for flow cytometric analysis of leucocytes. TruCount provided information on the absolute concentration of major leucocyte populations, while the 7-colour assay characterised a wider range receptor expression on these cells. Immediately after arrival, EDTA samples were placed on a Stuart Roller Mixer SRT6 until analysis. All incubation steps were carried out in the dark at room temperature.

3.4.3 TruCount assay

TruCount tubes contain a known number of fluorescent beads, allowing for precise calculation of the absolute count of a cell of interest with flow cytometry (**Equation 1**). Our antibody mix allowed calculation of absolute cells counts of $CD45^+$ (leucocytes), $CD19^+$ (B-lymphocytes), $CD16/56^+$ (Natural Killer cells), $CD3^+$ (T-lymphocytes), and $CD4^+$ and $CD8^+$ cells.

$$\text{Absolute count of } CD3^+ \text{cells}/\mu L = \frac{\text{Number of } CD3^+ \text{ cell events recorded}}{\text{Number of bead events recorded}} \; X \; \frac{\text{Number of beads per test tube}}{\text{Sample volume } (\mu L)}$$

Equation 1. Calculation of absolute count of $CD3^+$ cells using fluorescent beads in the TruCount assay

From each EDTA blood sample, 100µL was aliquoted and added to the bottom of a BD TruCount tube (340334, BD Biosciences) using a calibrated air displacement P200 pipette. 20µL of BD Multitest 6-Color TBNK reagent (644611, BD Biosciences) was deposited near the blood, on the side of the tube, ensuring no contact was made with the sample. The BD Multitest 6-Color TBNK consisted of the following antibodies, in buffer with 0.1% sodium azide:

- CD3 Fluorescein isothiocyanate (FITC)
- CD16 Phycoerythrin (PE)
- CD56-PE
- CD45-Per-Cy5.5

- CD4-PE-Cy7
- CD19 Allophycocyanin (APC)
- CD8-APC-Cy7

After vortexing the test sample using a Vortex Genie 2 (Scientific industries), the mixture was incubated for 30 minutes. Erythrocytes were lysed by adding 2mL of fresh lysis buffer 1x concentration, prepared using BD Pharm Lyse Lysing Buffer (10x Conc. 5075567, BD Biosciences) and Gibco distilled water (15230-147, Life technologies). After vortexing, and another 30 minutes of incubation, the samples were run using a BD LSRFortessa cell analyser (BD Biosciences, serial number: H647177E1001) until 3000 bead events were recorded, and data was extracted with BD FACSDiva Software. An example gating strategy for the TruCount assay is shown in **Figure 2**.

3.4.4 Seven-colour Fluorescence-Activated Cell Sorting assay

Multicolour flow cytometry was used to characterise the expression of a range of cell-surface receptors on T-lymphocytes. Three receptors analysed in this assay were also analysed with TruCount (CD3, CD4 and CD8) and some were not (CD45RA, CCR7, CX_3CR1 and CD28). By combining the absolute data of TruCount with the relative data of FACS, we were able to define the size of very specific subpopulations of T-lymphocytes.

For each EDTA sample, 100µL of fresh blood was aliquoted into Falcon® Round-Bottom Polystyrene FACS tube (352054, BD Biosciences). A cocktail of antibodies were added to the sample, as described in **Table 3**. The tube was vortexed and incubated for 30 minutes. 2ml of lysis buffer 1x concentrate (as prepared for the TruCount assay) was added and the tube was vortexed, then incubated for 30 minutes. The sample was then washed twice with PBS. Following each wash, the tube was centrifuged at 400G for 5 minutes and the supernatant was removed. Sample analysis and data acquisition was performed with the same machine and software as for the TruCount assay. The sample was run to 10,000 $CD8^+$ events. An example gating strategy is shown in **Figure 3**.

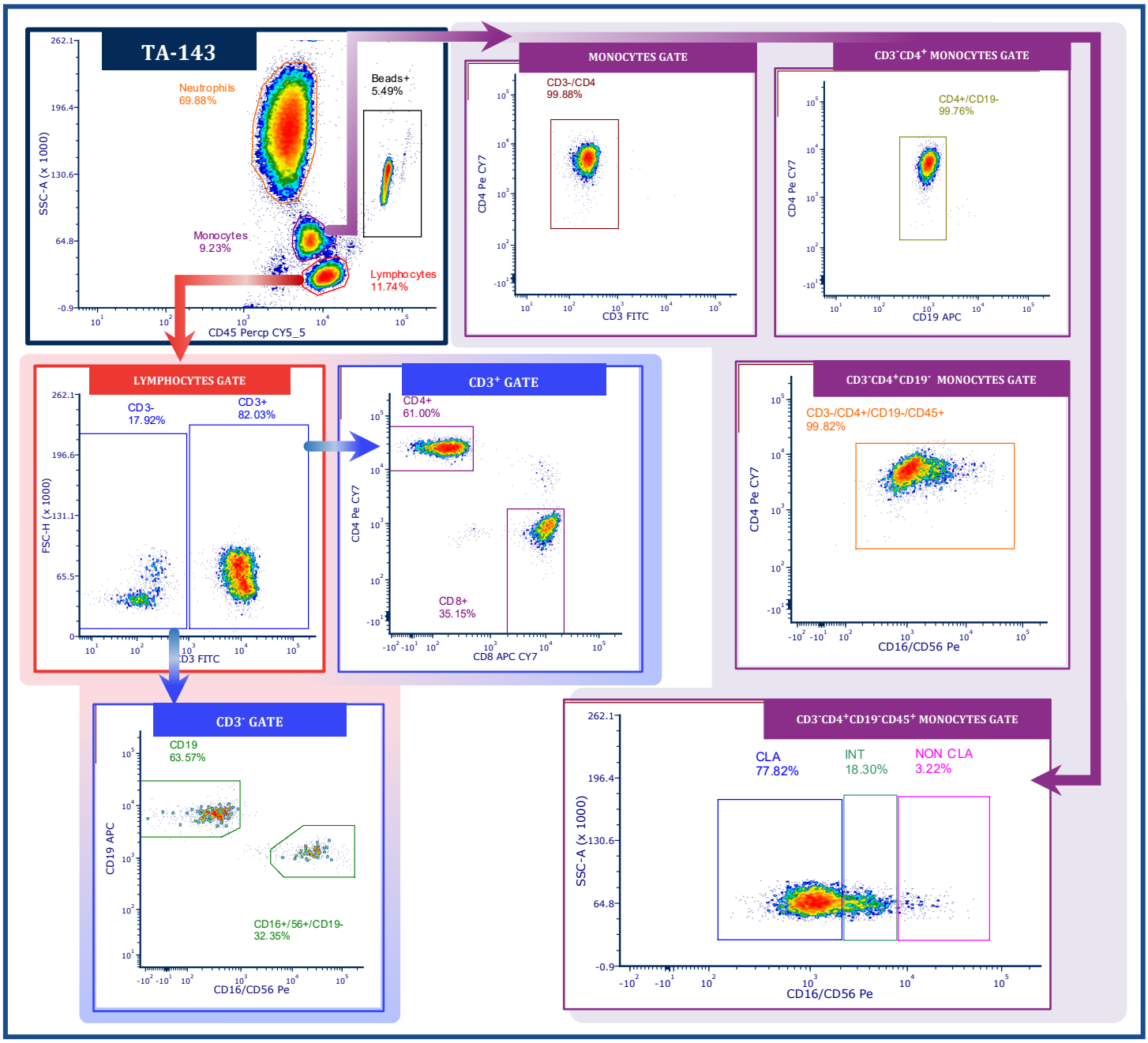

Figure 2 - TruCount assay gating strategy. Flow cytometric analysis of a representative patient, TA-143. Initially, lymphocytes, monocytes, neutrophils, and beads were gated in SSC-A/CD45 plot (CD45 eased the gating process since it is a marker for human leucocytes). Lymphocytes were sequentially gated into $CD3^+$ (T cells) and CD3- cells. $CD4^+$ and $CD8^+$ T cells were gated from $CD3^+$ cells, while B cells ($CD19^+$) and natural killer cells ($CD19^-CD16^+CD56^+$) were gated from $CD3^-$ cells. Several sub-gates were sequentially gated from the main monocyte population to ensure that gating was performed on specific $CD3^-CD4^+CD19^-CD45^+$ cells. Finally, monocytes were divided into classical, intermediate, or non-classical monocytes based on the abundance of CD16 expression.

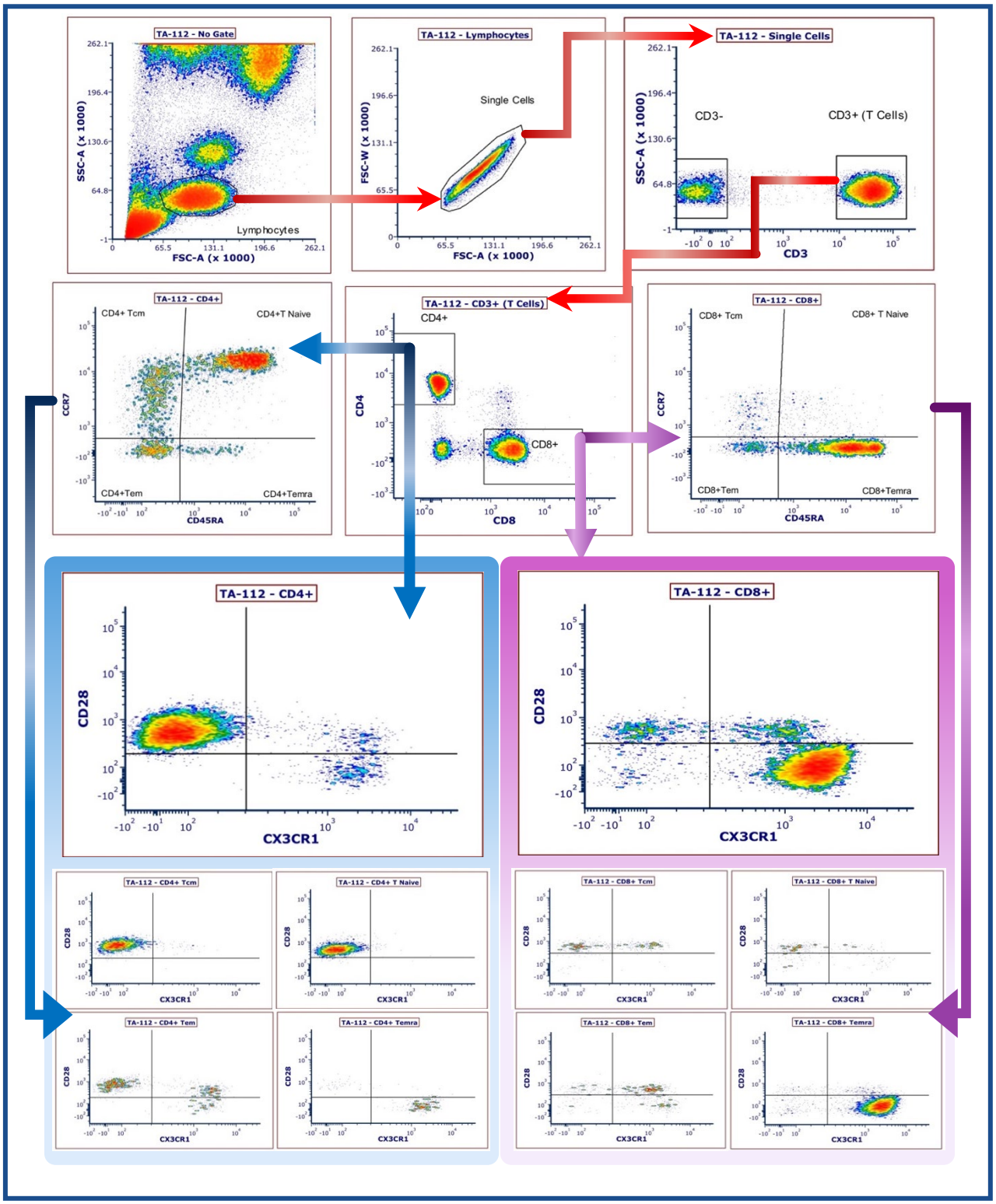
TA-112 - No Gate
SSC-A (x 1000)
FSC-A (x 1000)
Lymphocytes
TA-112 - Lymphocytes
FSC-W (x 1000)
Single Cells
TA-112 - Single Cells
CD3-
CD3+ (T Cells)
CD3
TA-112 - CD4+
CD4+ Tcm
CD4+T Naive
CD4+Tem
CD4+Temra
CCR7
CD45RA
TA-112 - CD3+ (T Cells)
CD4+
CD8+
CD4
CD8
TA-112 - CD8+
CD8+ Tcm
CD8+ T Naive
CD8+Tem
CD8+Temra
CD28
CX3CR1

Figure 3 – Gating strategy of 7-colour FACS. Data shown for a representative patient, TA-112. Lymphocytes were gated by FSC and SSC. $CD3^+$ lymphocytes were then subsequently gated into $CD4^+$ and $CD8^+$ T cells. Four subpopulations of $CD4^+$ and $CD8^+$ T cells (T_N, T_{CM}, T_{EM} and T_{EMRA}) were characterised by expression of CCR7 and CD45RA. CD28/CX3CR1 density plots were set for $CD4^+$ and $CD8^+$ T cells along with their subsets.

Reagents for 7-colour assay	Volume (µL)	Catalogue Number (Manufacturer)
CD3-PE	20	555333 (BD Biosciences)
CD4-V500	5	560768 (BD Biosciences)
CD8-FITC	20	555366 (BD Biosciences)
CD45RA- PE-Cy™7	5	337186 (BD Biosciences)
CCR7-BV421	5	353208 (BioLegend)
CD28-AF700	5	302920 (BioLegend)
CX3CR1-APC	5	341610 (BioLegend)

Table 3 – Antibodies used for the 7-colour flow cytometric assay. Catalogue number and manufacturer are given for each.

3.4.5 Telomerase Repeated Activation Protocol (TRAP) – quantitative polymerase chain reaction (qPCR)

Telomerase activity was measured using the Telomerase Repeated Activation Protocol (TRAP) – quantitative polymerase chain reaction (qPCR) assay. It is a PCR-based method that involves 3 basic steps:

1. Extension of an oligonucleotide by telomerase-mediated addition of telomeric DNA repeats
2. Subsequent PCR amplification of the extension products
3. Detection of telomerase products

Within 24 hours of collection, whole blood was centrifuged to isolate peripheral blood mononuclear cells which were cryopreserved at -80 C. The cells were subsequently thawed, lysed, and analysed using a validated qPCR protocol

Isolating PBMCs

Within 24 hours of collection, whole blood was centrifuged at 800G for 15 minutes to isolate PBMCs. These were cryopreserved at -80°C until being thawed for cell lysis and analysis.

Positive control

The human T-cell leukaemia cell line 1301 (Sigma-Aldrich) was used as a positive control during qPCR, as these cells have unusually long telomeres.

Cell lysis

Cryopreserved PBMCs (or 1301 cells for the positive control) were thawed and added to 9mL of phosphate-buffered saline (PBS; pH 7.2; 20012027, Gibco). This suspension was mixed, and 10µL was pipetted onto each side of a haematocytometer for counting. The cells were counted on each side and an average taken. The volume of suspension which would contain $2x10^6$ cells was calculated, pipetted out and centrifuged at 300-400G for 5 minutes to form a pellet.

The supernatant was then removed, and 500µL of PBS was added. The contents were resuspended and added to an Eppendorf tube. These were centrifuged for 8 minutes at 300-400G, with the tubes turned halfway through, to ensure complete lysis. The supernatant was carefully removed without disturbing the pellet of PBMCs. 40mL of lysis buffer 1x concentrate was added to the pellet to achieve a cell concentration of 50,000/µL. Lysis buffer was produced with BD Pharm Lyse Lysing Buffer (10x Conc. 5075567, BD Biosciences) and Gibco distilled water (15230-147, Life technologies). This was mixed well, then stored on ice for 30 minutes. This was then centrifuged at 4^{o}C for 20 minutes at 12,000G. These were aliquoted into 5µL PCR tubes and stored at -80^{o}C until qPCR analysis.

TRAP-qPCR

Samples (and positive controls) were removed from the freezer, and briefly centrifuged again. One 5µL tube for each sample (including positive control) was heat inactivated by incubating at 85 degrees for 10 minutes.

45µL of nuclease-free water was added to all samples, including the positive control, to achieve a cell concentration of 5,000/µL. The positive control was then twice diluted again 5-fold with nuclease-free water, to give final concentrations of 5,000, 1,000 and 200 cells/µL for the positive control.

2µL of sample were added to each well of a 96-well qPCR plate. Four replicates of each active sample and positive control were used, along with two replicates each for heat inactivated samples and lysis-buffer only samples.

The qPCR master mix was made with the reagents shown in **Table 4**, with volume shown per sample. This was made in batches to reduce pipetting error. The telomerase primer stock solution contained 10µL of 100ng/µL stock of both TS (telomerase substrate) primer (5'-AATCCGTCGAGCAGAGTT) and ACX primer (5'-GCGCGGCTTACCCTTACCCTTACCCTAACC-3') diluted in 80µL nuclease free H_2O.

18µL of master mix was added to each well. The plate was gently vortexed using a Vortex Genie 2 (Scientific industries), then centrifuged at 300-400G. The pPCR was then run with conditions as shown in **Table 5**, using a QuantStudio 7 Flex qPCR machine.

Validation

This protocol was validated through analysis of 8 quantities of human T-cell leukaemia cell line 1301 and generation of a standard curve. Serial 1:5 dilutions were used to give cell numbers ranging from 1 million to 13. The standard curve showed strong correlation ($r^2 = 0.94$) and a slope value of -3.3, suggesting 100% efficiency (**Figure 4**). We found that telomerase activity was lower in PBMCs from patients in TACTIC than in the positive control (**Figure 5**). We therefore exclusively used 10^6 cell samples for this assay with PBMCs from patients.

Limitations

- Quantitative measurement of telomerase activity requires comparison to internal standards and reference cell lines.
- Technical issues: effect of PCR inhibitors such as heparin.
- changing composition of peripheral blood leukocytes
- Variability in counting cell input
- Effect of cell cryopreservation on TA variability
- TA measured following lysis of complete cells, which might not represent the situation in vivo where enzymatic components of telomerase could be located in separate compartments of the cells.

Reagent	Volume per sample
Nuclease-free H_20	4.8µL
Telomerase primer stock solution	1.6µL
FastStart SYBR Green Master 2x concentration (Sigma-Aldrich #46734001)	10µL
EGTA (10mM)	2µL

Table 4 – Reagents used for TRAP-qPCR assay.

Step	Temperature (°C)	Time	No. cycles
Telomerase activation	37	15 minutes	1
Telomerase inactivation, polymerase activation	95	10 minutes	1
Denaturing	95	20 seconds	36
Annealing	52	20 seconds	
Extension	72	45 seconds	
Data acquisition	Plate read		
Melt curve analysis	95	15 seconds	1
	60	1 minute	
	95	15 seconds	
	Plate read		

Table 5– Conditions for TRAP-qPCR assay.

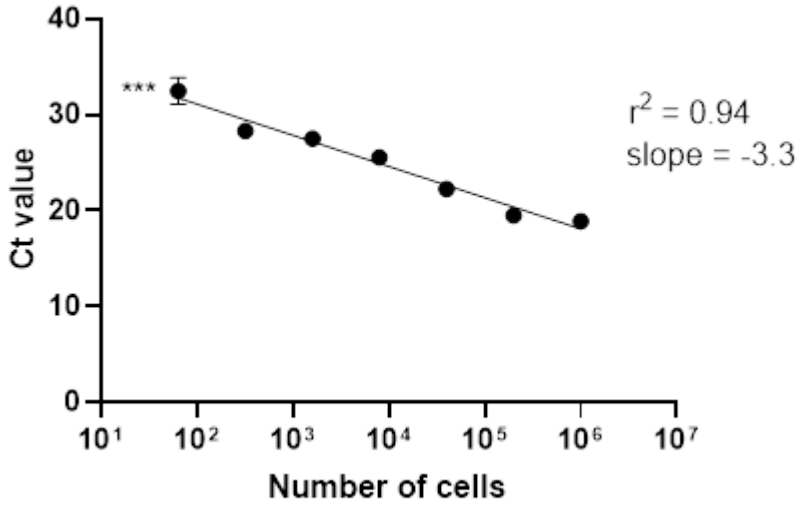

Figure 4. Standard curve for TRAP-qPCR assay using human T-cell leukaemia cell line 1301. Shown are 8 serial 1:5 dilutions, starting at $1x10^6$ cells. There is a strong correlation (r^2 = 0.94; p<0.001), and the slope value of -3.3 suggests 100% efficiency of the PCR.

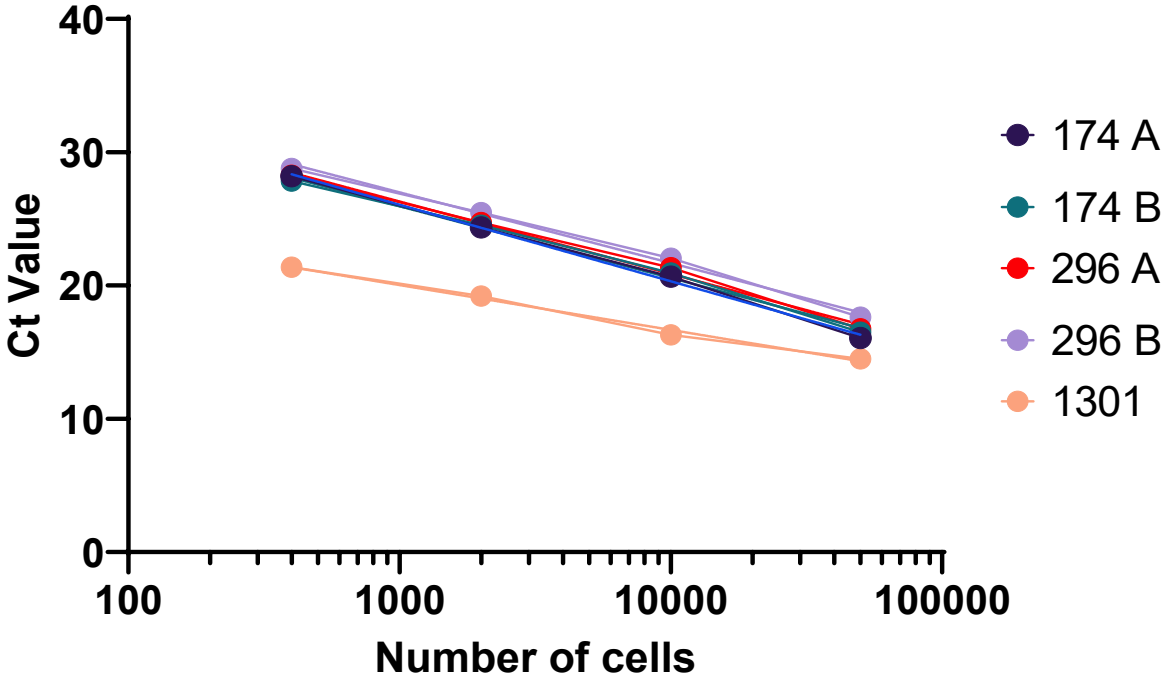

Figure 5. Reproducibility of TRAP-qPCR results in TACTIC patients and positive control. Serial 1:5 dilutions (starting with 50,000 cells) for the positive control (cell line 1301) and PBMCs from TACTIC patients 174 and 296 (two samples for each, A and B). In PBMCs from TACTIC patients, there was lower telomerase activity and a steeper

slope of the calibration curve. All standard curves showed excellent reproducibility with r^2 ≥0.99. Slope value for positive control was -3.37, and slope values for TACTIC patient samples were -5.74 (174A), -5.38 (174B), -5.43 (296A) and -5.31 (296B). Error bars are too small to be shown.

3.4.6 TBARS measurement

Lipid peroxidation oxidative stress was measured using thiobarbituric acid-reactive substances (TBARS) Assay Kit, Cat# KGE013, R&D, Minneapolis, USA. For precipitating interfering proteins and other substances, all the plasma patient samples were treated with trichloroacetic acid (TCA), with 1:1 ratio of 200ul plasma mixed with 200 ul of TCA. After 15 minutes incubation at room temperature, the samples were centrifuged at 12000 x g for 4 minutes, then 150ul of supernatants were incubated with 75ul of thiobarbituric acid (TBA) on a thermoshaker at 45 °C for 3 hours. The resulting color was measured spectophotometrically by determining the optical density of each well containing the patient plasma sample using Varioskan™ LUX (Thermo Scientific) multimode microplate reader at 532 nm wavelength.

3.4.7 Systemic Inflammation

C reactive protein (CRP) is an acute phase protein produced by the liver in response to pro-inflammatory cytokines namely interleukin 6 (Figure 6). CRP is a well-established biomarker of systemic inflammation and is measured by the highly sensitive assay – hsCRP. In patients without acute infections or inflammatory diseases, hsCRP levels remain stable with variability over years comparable to cholesterol levels[165]. Its stability during long term frozen blood storage and availability of standardised assays have promoted its use in clinical and research settings. CRP is found in atherosclerotic plaques, bound to low density lipoproteins [166].

Studies have shown that peak CRP levels in patients with MI are directly associated to infarct size and left ventricular function [167], [168] at follow up. Hence, peak CRP may help to estimate the probability of unfavourable outcomes in MI patients. Given that CRP is produced by the liver because of an upstream cascade and in relation to the degree of cell migration to the infarct zone, it may take up to 48 hours to reach peak levels. The inflammatory phase post MI may extend up to several weeks and

hsCRP at one month is a recognised predictor of recurrent ischemic events [169] [170][171]and heart failure [172]. In the chronic phase, low-grade inflammation may increase susceptibility to adverse events. In a pre-specified analysis of Pravastatin or Atorvastatin Evaluation and Infection Therapy–Thrombolysis in Myocardial Infarction 22 (PROVE IT–TIMI 22) study (PROVE-IT TIMI 22), patients with elevated hs-CRP (≥2.0 mg/L) 30 days after acute MI had increased risk for recurrent MI and death than those with lower hs-CRP levels irrespective of LDL-C reduction [173] In apparently healthy patients, elevated baseline CRP appears to be associated with increased cardiac risk. The JUPITER trial [Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin] demonstrated that, among patients with optimal LDL-C levels, targeted treatment for primary cardiovascular prevention with statin therapy in patients with elevated hs-CRP levels (≥2.0 mg/L) resulted in a significant reduction in cardiovascular events. CRP is therefore considered a robust marker to identify high risk patients who are more likely to respond to anti-inflammatory therapy. However, mendelian randomisation studies of polymorphisms in the CRP gene showed that CRP concentration is unlikely to be causal factor in coronary heart disease [174]. In a large meta-analysis of over 160000 subjects without a history of vascular disease, the association of CRP concentration with CAD and stroke is dependent on traditional risk factors.

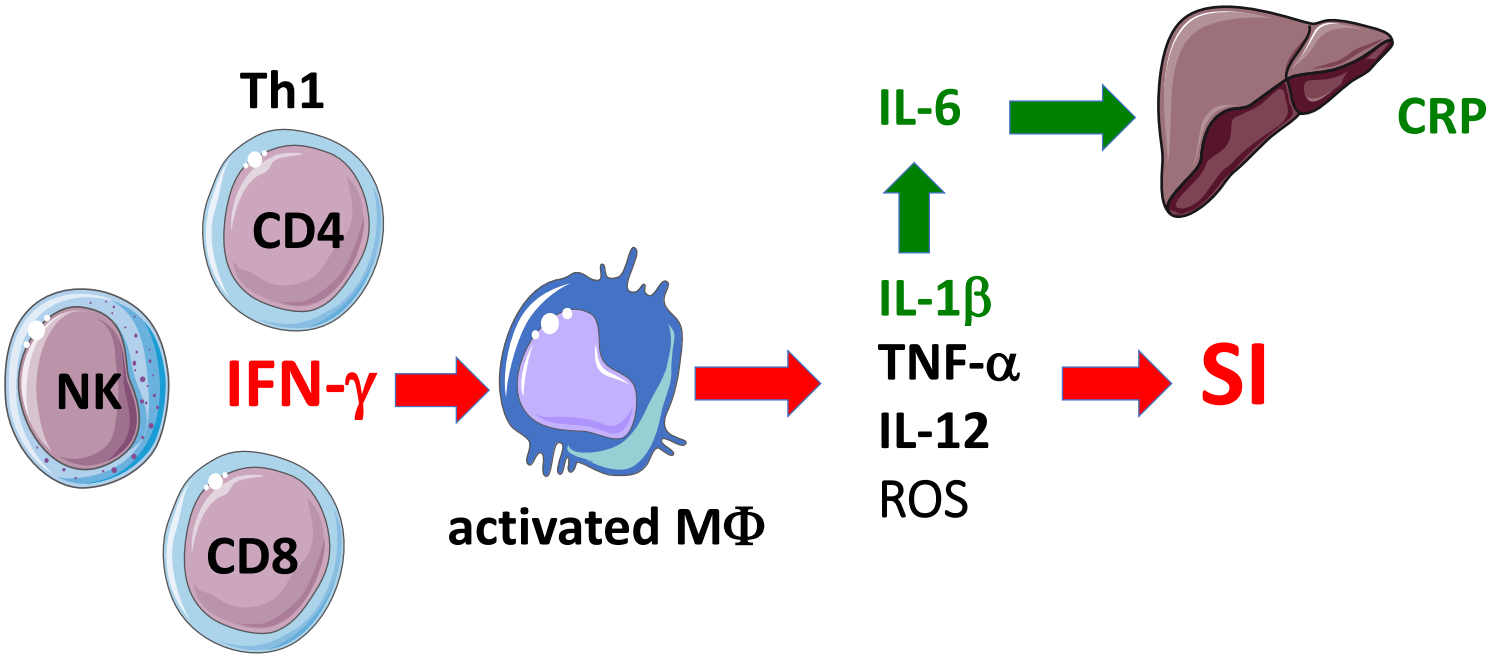

Figure 6. CRP is a downstream inflammatory biomarker. NK= natural killer cells, IFN-γ= interferon-gamma, MΦ= macrophages, IL= interleukin, TNF-α= tumor necrosis factor alpha, ROS= Reactive oxygen species, CRP= C-reactive protein, SI= systemic inflammation

3.4.8 Seropositivity to cytomegalovirus

The effect of baseline cytomegalovirus (CMV) seropositivity was correlated with study outcomes using an exploratory analysis.

3.5 Endothelial function

3.5.1 Biological function of endothelium

The endothelium is a monolayer of cells covering the internal lumen of all blood vessels. It has key functions:

- Vascular tone and blood flow regulation
- Anticoagulant surface
- Vascular homeostasis and repair
- Control of fluid and molecules between blood and tissues

Vascular tone is controlled by the balance between endothelium-derived vasodilators namely nitric oxide, prostacyclin and C-type natriuretic peptide, and vasoconstrictors such as endothelin[175]. Nitric oxide (NO) is produced from L-arginine by endothelial NO synthase and it circulates to adjacent smooth muscle cells to mediate vasodilation. In the coronary artery, the microcirculation autoregulates the delivery of blood to the myocardium over a wide range of perfusion pressures and myocardial oxygen demand.

3.5.2 Endothelial dysfunction

Endothelial dysfunction is characterised by a change in phenotype from NO-mediated inhibition of cellular processes to activation by redox signalling[175]. By interacting with circulating leukocytes and platelets, this switch may trigger the start of an inflammatory response. Subendothelial entry of apolipoprotein B-containing lipoproteins are key initiating steps in initiating atherosclerosis. Oxidative stress and the vessel wall's proinflammatory condition are related. Reactive oxygen species are produced in large quantities by mitochondria and during oxidative phosphorylation, the synthesis of ROS

and mitochondrial superoxide dismutase is carefully regulated[175]. This equilibrium is disturbed by hypoxia, obesity, type 2 diabetes and dyslipidemia [176], [177]. ROS promote the expression of chemotactic molecules and adhesion molecules, including intercellular adhesion molecule-1 and vascular adhesion molecule-1 (macrophage chemoattractant peptide 1). Reduced eNOS activity has been linked to increased CRP levels [178]. Endothelial dysfunction has been identified as a predictor of adverse clinical outcomes in patients with CAD[179].

3.5.3 Measures of endothelial function

Endothelium-dependent vasodilatation can be assessed by invasive and non-invasive methods. The gold standard method of evaluating coronary endothelial function is intracoronary administration of endothelial dependent vasodilatory agents and quantitative angiography [180].

In the setting of normal endothelial function, intracoronary infusion of acetylcholine triggers NO-mediated vasodilation of epicardial coronary arteries. In contrast, acetylcholine provokes vasoconstriction through a direct action on smooth muscles in the presence of endothelial dysfunction.

Non-invasive assessment of peripheral arterial endothelial function is based on the principle of flow-mediated dilatation (FMD). The endothelium can provoke vasodilation in response to shear stress and increased blood flow. Evaluation of FMD of forearm arteries is a recognised method of measuring peripheral vasodilator response. The technique involves baseline and post artery occlusion ultrasound measurement of brachial artery dimensions and doppler estimation of flow. The impact of the resulting hyperaemic flow on brachial arterial size is assessed after 5 minutes of arterial obstruction. Despite good correlation with coronary artery endothelial function, forearm FMD measurement is technically challenging and is limited by interobserver variability and reproducibility [181]. Studies using mostly FMD measurements have shown improved endothelial function following dietary change or pharmacological intervention [182]–[184]

3.5.4 EndoPAT device

The EndoPAT device (Itamar Medical Ltd, Caesarea, Israel) was used to evaluate endothelial function by automatically calculating the reactive hyperaemia index (RHI) in an operator-independent manner. RHI is the post to pre occlusion PAT (peripheral arterial tone) signal ratio in the occluded arm relative to the same ratio in the control

arm adjusted for baseline vascular tone. Hence, compared to FMD, EndoPAT allows correction of potential measurement-induced changes in systemic haemodynamics, e.g., due to alterations in the autonomous nervous system. There was a linear relationship between the RHI and FMD during the same episode of reactive hyperaemia ($r=0.5$, $p<0.0001$) in 89 patients with chest pain[185].

RHI has been shown to reflect NO-bioavailability [186]. RHI lower than 1.35 was 80% sensitive and 85% specific in identifying patients with coronary endothelial dysfunction [187]. In 329 patients with chest pain and low risk stress test, a natural logarithmic scaled RHI < 0.4 was independently predictive of long term adverse cardiovascular outcomes [188].

Patients were seated in a quiet and comfortable room for at least 15 minutes before the start of the test. Blood pressure was measured from the patient's control arm. The PAT probes were inserted in each index finger and a blood pressure cuff placed on the upper test arm. Both PAT probes were connected by pneumatic tubes to an inflating device controlled by a computer running the EndoPAT software.

The PAT signals from both test and control arms were visualised and inspected for strength and stability. Baseline signals were recorded for 5 minutes. The blood pressure cuff was rapidly inflated and maintained at supra-systolic level (at least 60 mmHg above systolic blood pressure) for 5 minutes. Total cessation of blood flow to the test arm was verified by the total absence of the PAT signal. The blood pressure cuff was rapidly deflated and post occlusion PAT signals recorded for 5 minutes.

Following the recording period, the RHI was obtained using the automatic analysis function on the software (**Figure 7**). Occlusion borders are reviewed and adjusted manually if appropriate.

Data on the reliability of RH-PAT are scarce. In a study assessing reliability of repeated RHI measurements using the EndoPAT device in healthy volunteers and stroke patients, repeated measurements on day-to day (intraclass correlation coefficient ICC 0.56) showed modest but better reliability than on same day (ICC 0.25). This suggests a carryover effect if sufficient time between measurements is not applied for the vessels to regain steady vascular state [189]. This observation was also demonstrated in a study of 18 patients with stable CAD[190].

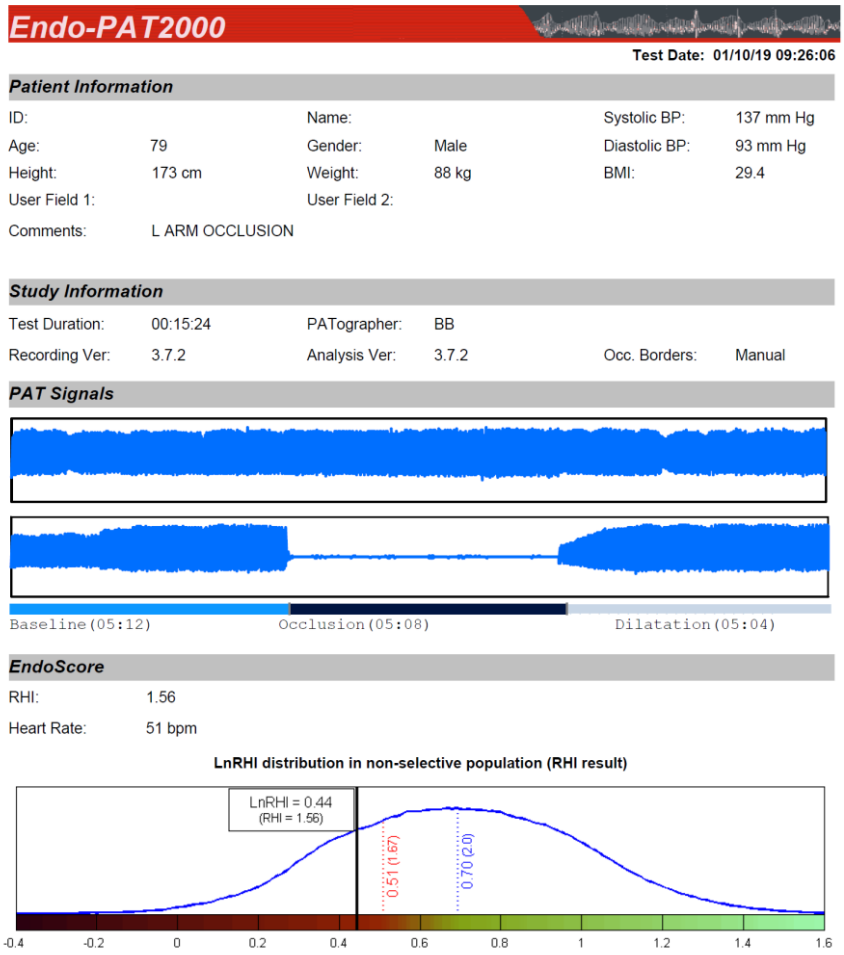

Figure 7. Automatic analysis of RHI (reactive hyperaemia index) using pre and post occlusion PAT signals in control and test arms.

3.6 Cardiac function

Measurement of NT-proBNP and transthoracic echocardiography (TTE) were performed at baseline and 12 months to assess cardiac function. Cardiomyocytes from mice used in preclinical studies have indicated that telomerase deficiency and short telomeres are crucial for the emergence of heart failure [191]. Patients with chronic heart failure have shorter telomere length compared to age and gender-matched controls with incremental attrition according to the presence and extent of CAD.

Patients underwent 2-dimensional (2D) transthoracic echocardiography at baseline and 12 months, using the Phillips Affiniti 70 and GE Vivid E95 ultrasound system. Measurements were performed in either a minimum of three or five cardiac cycles

depending on the underlying cardiac rhythm (sinus rhythm or atrial fibrillation, respectively).

All echocardiographic images were digitally stored in DICOM format and analysed offline using TOMTEC (TOMTEC Imaging Systems, Unterschleißheim, Germany) software at the James Cook University Hospital Echocardiography Core Laboratory, by echocardiography accredited cardiology registrars (SV and BB).

Using the parasternal long axis view, 2D linear measurements of the left ventricular cavity dimensions were performed. Non-foreshortened apical 4- and 2- chamber views with a good endocardial definition were used for left ventricular volumetric assessment. Left ventricular ejection fraction was then quantified using the modified Simpson's biplane measurement. Transmitral pulsed wave Doppler from the mitral valve leaflet tips in the apical 4-chamber view was used for the measurement of peak early (E)€ and late (A) diastolic filling velocities including the E-wave deceleration time. Additionally, lateral and septal peak systolic (S'), early (E') and late (A') annular diastolic velocities were measured using the Tissue Doppler Imaging (TDI) at the lateral and septal mitral valve annulus in the apical four-chamber view. Left ventricular end-systolic global longitudinal strain (GLS) was measured using TOMTEC semi-automated software (AutoSTRAIN©) after selection of non-foreshortened apical 4-, 2- and 3-chamber views with reasonable image quality and complete R-R cycles. Pulse waved spectral Doppler in the apical 5-chamber view was then used to allow the measurement of aortic valve closure time (AVC) for end-systolic GLS quantification. If automated endocardial tracings were deemed unsuitable adjustments to these tracings were made to allow accurate GLS measurement. If tracking of the endocardium was unsatisfactory, GLS was not measured.

3.7 Trial procedures

3.7.1 Identification and screening of participants

Potential participants were identified by the clinical team at The James Cook University Hospital following admission to hospital with ACS and having agreed to undergo coronary angiography or having already had an angiography that confirmed CAD. Patients were also identified following discharge.

3.7.2 Recruitment and consent procedures

A delegated member of the study team sent invitations to prospectively qualified participants. After the clinical research team member had fully given the patient time to consider and ask any questions, the patient was asked for written consent to participate in the trial. The process emphasized that the standard of care of the patient would be unaffected by the patient's decision. Written informed permission was obtained before any trial-specific procedures and before randomization.

The original signed consent forms were kept in the Investigator Site File, with a copy in the clinical notes and a copy given to the patient. Participants specifically consented to their GP being informed of their participation and to their samples being stored within a Biobank for future analysis. Patients had the right to exit the trial at any point and without giving a reason. General Practitioners were notified of each patient's decision to participate with their expressed consent. All patients who were offered to participate had their information entered into a screening log, along with any explanations they provided for declining or not being randomly assigned (e.g. not meeting eligibility criteria for the trial). An eCRF was created and a participant ID number was issued only to individuals who were randomised.

3.7.3 Eligibility assessment and review of coronary angiogram

All individuals were required to have a formal diagnosis of an ACS within 6 months and to undergo coronary angiography as part of standard care, which confirmed severe coronary heart disease (at least one epicardial artery stenosis of 70%). The Principal Investigator or a sub-Investigator on the delegation log confirmed patient's eligibility, including review of prior coronary angiography. Patients who provided consent but did not match the study eligibility requirements before randomisation were deemed "screen failures" and were removed from the experiment; no more data were gathered.

3.7.4 Randomisation

Patients were randomly assigned to each group using the Sealed Envelope Ltd method with a minimization technique to assure baseline comparability. The minimisation scheme accounted for:

- Gender (male or female).
- Type of ACS (STEMI or NSTEMI).
- $CD8^+$ T_{EMRA} (high > 45% or low ≤45) at baseline.

Using the 24-hour, central, secure, web-based randomization method with concealed allocation, delegated and trained members of the study team randomly assigned the eligible patients. In a 1:1 ratio, eligible patients were randomly assigned to receive either the research intervention, TA-65MD®, or a placebo (control arm).

3.7.5 Blinding

The clinical study team, treating professionals, and patients were blinded to the patient's assignment to either TA-65MD® or placebo. Blinding was maintained in the randomization system, on IMP labels, and on Electronic Case Report Forms (eCRFs). With the exception of the trial Data Manager, all Newcastle Clinical Trial Unit (NCTU) personnel and the statistics team were blinded in order to facilitate accurate reporting to the independent data monitoring committee. A unique identifying code that was associated with the mechanism of study randomization was used to identify the IMP. To guarantee that the IMP was blinded, TA-65MD® and the matching placebo were identical and supplied in the same package.

3.7.6 Unblinding

In the event of medical emergencies, where patient treatment necessitated knowledge of the randomisation allocation, unblinding was implemented. Permission to reveal trial subject identities was only granted to selected personnel. Any unblinding was promptly reported to the PI, who also contacted NCTU to alert the Independent Data Monitoring and Ethics Committee and the appropriate authorities. The primary trial analysis was completed prior to unblinding.

3.7.7 Baseline assessments

Baseline evaluations were carried out at least 24 hours after the ACS diagnosis to reduce the impact of acute inflammation on trial results. Baseline blood samples were obtained a month or less before the commencement of the study therapy. Blood samples needed for flow cytometry and telomerase activity were delivered to Newcastle University on the same day and processed in accordance with the TACTIC laboratory handbook. The randomisation approach employed the baseline CD8+ TEMRA result as a stratifying variable. Before delivery, the laboratory at James Cook University Hospital prepared and frozen additional study blood samples (serum and plasma), as well as the plasma needed for the oxidative stress assay. Biological samples will be stored for up to 5 years after the end of the trial at which point, any unused samples will be destroyed.

3.7.8 Study visits

Where possible, baseline assessments, randomisation and study drug initiation were performed during the patient's index hospitalisation. This arrangement suited STEMI patients who stayed in hospital for at least 48 hours after coronary angiography. Most patients presenting with NSTEMI were discharged within 24 hours after coronary angiography and therefore were scheduled for an outpatient 'baseline study visit' at their earliest convenience. After baseline assessments were completed, those patients were invited to a 'randomisation and drug initiation' visit within a month. This arrangement was made to avoid patients waiting for several hours for the result of $CD8^+$ T_{EMRA} which was necessary for randomisation. Following randomisation, patients were advised to start study medication immediately and continue taking this twice daily for 12 months. Patients returned to site for study visits at months 1, 3, 6, 9 and 12 (**Table 6**). At each visit, patients were offered private taxis or had their transport costs subsidised.

	Baseline[a]	Start of study drug (Day 1)	1m (weeks 3-5)	3m (weeks 11-15)	6 m (weeks 24-28)	9 m (weeks 37-41)	12 m (weeks 50-54)
ENROLMENT							
Eligibility screen	X						
Informed consent	X						
ASSESSMENTS							
Blood pressure	X		X	X	X	X	X
Capillary glucose	X		X	X	X	X	X
Physical assessment of height and weight	X						
Venous sample (5mL) CMV IgG	X						
Venous sample (4mL) CD8 TEMRA immunosenescence (primary outcome)[b]	X				X		X
Venous sample (36 mL) CD8 and CD4 immunosenescence (secondary outcomes), Telomere length, Telomerase activity	X				X		X
Venous sample (4 mL) Oxidative stress, future use	X				X		X
Venous sample (5 mL) Future research use- optional consent	X				X		X
Venous sample (5mL) hsCRP, NT-proBNP	X				X		X
Endothelial function (EndoPAT)	X				X		X
Echocardiography	X						X
INTERVENTIONS							
Randomisation- stratified	X						
Dispensing of IMP		X	X	X	X	X	X
Return of unused IMP/ drug adherence			X	X	X	X	X
Adverse events evaluation			X	X	X	X	X

Table 6. Study visit schedule, assessment and intervention[127].

3.7.9 Impact of COVID-19 and protocol amendments

During the COVID-19 pandemic, due to government guidelines, patients were unable to attend site for face-to-face visits as they were considered vulnerable due to heart disease, other comorbidities and many due to their age (>70 years). Their study visits were carried out remotely, over the telephone, by a delegated member of the site team. The period that some patients received IMP was extended so that primary and secondary outcome data could be collected when it was safe for the patients to return to site for their final visit. Patients who had not yet attended their 6-month appointment received portable blood pressure and blood glucose monitors to use at home to record their readings in accordance with the pandemic's planned course of action.

3.6 Study intervention

3.6.1 Intervention under study: TA-65MD®

Cycloastragenol 1.8% is a triterpenoid extracted from the roots of Astragalus species and is the active component of TA-65MD. It was detected in an empirical screening of plant extracts and chemicals used in traditional Chinese medicine. TA-65MD is a telomerase activator. Patients in the intervention group received 8mg of TA-65MD twice daily. The matched placebo was created to be identical to TA-65MD® in terms of appearance, flavour, aroma, labelling and packaging.

3.6.2 Investigational Medicinal Product and Packaging

For the purposes of this trial, TA-65MD® 8 mg BD (T.A. Sciences. Inc, New York), and the matched placebo were treated as IMP. T.A. Sciences. Inc, New York formulated and manufactured the IMP; QPS Netherlands BV was responsible for packing, labelling and supplying the IMP for the trial. Study drugs were securely stored at The James Cook University Hospital Pharmacy with temperature controls and continuous temperature monitoring. A supply of IMP was allocated to participants upon receipt of a study specific prescription.

3.6.3 Safety data

According to the Federal Food, Drug, and Cosmetic Act, which is overseen by the United States Food and Drug Administration (FDA), an independent expert panel found that TA-65MD is Generally Recognized as Safe (GRAS) for use in a medical food. The designation of "Generally Recognized as Safe" was supported by significant animal and human clinical evidence from T.A. Sciences, Inc. The safety of TA-65MD was evaluated in a 1-year observational trial including 114 adults[192]. No adverse events associated with TA-65MD were observed at doses up to 50 mg daily. A randomised placebo-controlled trial including 117 adult volunteers also evaluated safety, and there were no abnormalities of liver, renal or metabolic functions over the course of a year [163]. The potential for TA-65MD to activate telomerase poses a theoretical risk of activating cancerous cells but this has not been proven[160] [193] [194]

A controlled in vivo cancer study using 5ml/kg of CAG or sham medication given by oral gavage for up to 40 days in mice xenografted with 4 different human tumours (lung, colon, breast and prostate) suggested a trend toward tumour growth retardation in two cancer types and no statistically significant negative effect on body weight or tumour size in other cell lines (Internal T.A. Sciences document 2008).

3.6.4 Known side effects

There are no known severe side effects associated with TA-65MD. It may have an impact on blood pressure and blood sugar levels (Clinical Investigator Brochure, T.A. Sciences 2017) although this has not been proven in trials. In an observational study, two subjects who voluntarily increased their daily TA-65MD® consumption to 100 mg/day—a level twice the intended dose for this observational study—reported feeling "anxious" shortly after. The feelings disappeared in both subjects when daily consumption was reduced to 50 mg/day. [163]. No product related toxicity was reported in two randomized placebo-controlled study over 1 year study duration[164], [195]

3.7 Administration and adherence

Participants were advised to take the IMP immediately following the completion of all baseline assessments and randomisation. TA-65MD 8 mg or matched placebo was taken as one capsule twice daily (morning and evening) for 12 months. Patients were prescribed IMP at baseline, 1, 3, 6 and 9 months following the start of the intervention. Each participant's adherence was determined based on the number of returned capsules at each visit.

3.8 Withdrawal of participants

Participants were informed of their right to withdraw from the trial at any point for any reason, and without justification. Participants were withdrawn for the following reasons:

- Inter-current illness preventing the participant from completing study procedures,
- Unacceptable side effects caused by the IMP
- The participant became ineligible between randomisation and start of study treatment

- Suspected unexpected serious adverse reactions occurred

- Protocol violations

- Any other reason that would compromise participant safety or the validity of the results

Patients were asked upon withdrawal:

- To return all unused trial drug
- Whether further data can continue to be collected from their medical records
- Whether samples collected for future research may still be analysed

3.9 Pharmacovigilance

All AEs were documented in the participants' medical notes and electronic Case Report Forms from the day of randomisation until the last visit or withdrawal. AEs considered related to the IMP were followed until resolution, a stable outcome or death. All AEs were assessed for severity, causality, expectedness, and seriousness by an Investigator. Causality of each AE was determined by a medically qualified individual using the definitions listed in **Table 7**.

Relationship	Description
Unrelated	The event is not considered to be related to the IMP and there is no evidence of any causal relationship.
Unlikely to be related	The event is considered unlikely to be related to the IMP; there is little evidence to suggest a causal relationship or there is another reasonable explanation for the event (e.g. the participant's clinical condition, other concomitant treatment).
Possibly related	Although the relationship to IMP cannot be ruled out, there is some evidence to suggest a causal relationship. However, the influence of other factors such as the nature of the event, the underlying disease, concomitant medications or temporal relationship make other explanations plausible.
Probably related	The temporal relationship and an absence of a more likely explanation suggest that the IMP is the most likely cause.
Definitely related	The known effects of the IMP indicate this to be the most likely cause/ there is clear evidence to suggest a causal relationship, and other possible contributing factors can be ruled out.

Table 7. Causality of Adverse events.

The severity of each AE was determined using the definitions in **Table 8**.

Grade	Description
Grade 1	Minor AE that does not need medical attention. It may be symptomatic but only a minor, temporary incident not requiring medical attention. Asymptomatic laboratory or radiographic findings are also included.
Grade 2	An AE which may require some medical intervention (local/non-invasive) and which is symptomatic to patient. May affect activities of daily living.
Grade 3	Significant symptoms reported, requiring medical intervention and possibly requiring hospitalisation. Medically significant and likely to be significantly affecting activities of daily living.
Grade 4	An AE that requires urgent intervention or may have life-threatening consequences.
Grade 5	Death related to the AE.

Table 8. Definition of Adverse Event severity

3.10 Data Management

Study data were entered into each patient's medical notes and then entered onto computerised Case Report Forms. The Sealed Envelope Ltd. Red Pill system was used to develop the eCRF. The information on the eCRF and medical notes were corroborated. Patients were identified using a specific study ID, and all data sent to NCTU included only study ID, date of birth, gender, and ethnicity.

3.11 Statistical considerations

3.11.1 Sample size

A pragmatic choice was made to obtain a sample size of 90 patients. This is the minimum threshold for estimating parameters in pilot studies [196]. The parameter estimates will guide a multi-center clinical trial [127]

3.11.2 Summary of Study variables

Continuous variables were summarised using the following descriptive statistics: (non-missing sample size), mean ± SD (or median ± IQR if data are skewed), maximum and minimum. The frequency and percentages (based on the non-missing sample size) of observed levels were reported for all categorical data. All summary tables were structured with a column for each treatment in the order (Treatment, Control) and were annotated with the total population size relevant to that table/treatment, excluding missing observations. All efficacy analyses and 95% confidence intervals were for the purpose of guidance and the focus was on the estimated effects rather than statistical significance. All analyses were performed on a PC running Microsoft Windows 10, and the statistical programming code R was used for the analysis.

3.11.3 Primary analysis

The proportion of terminally differentiated $CD8^+$ effector memory cells ($CD8^+$ T_{EMRA}) was calculated from the total number of peripheral blood $CD8^+$ T cells in each blood sample, thus, it was treated as a continuous outcome. Therefore, a linear mixed effect model assuming Gaussian distribution and identity link with minimising factors as predictors was used for the primary analysis using intention-to-treat principles to quantify change in proportion of $CD8^+$ T_{EMRA} from baseline. Only the primary endpoint was used to characterise patients who had missing data, with further sensitivity analyses to check the impact of the missing data. The interaction effect between time

points and the trial arm variables in the model shows the main effect of treatment (TA-65MD® – Placebo) over time. If the treatment effect is significant, it shows the proportion of the $CD8^+$ T_{EMRA} has changed over time using TA-65MD® compared to Placebo.

3.11.4 Secondary outcome analysis

The secondary outcomes were summarised by treatment group. Specifically, N, mean ± SD, median ± IQR, minimum and maximum were reported. Continuous secondary outcomes were analysed using linear mixed-effects models to consider the follow-up time effect and intra-patient correlation. Negative binomial models were used for count data and logistic regression models for binary data. All models accounted for stratification variables used in the randomisation scheme. The coefficients and their associated confidence intervals were reported for each variable included in the models.

3.11.5 Timing of Analyses

All data were analysed at the end of the study. No interim analysis was conducted for this trial. Where laboratory outcomes could not be measured due to the impact of COVID on access to expertise, these were analysed later and added to the final dataset for the trial.

3.11.6 Covid-19 restrictions

Due to the national lockdown starting from 23rd March 2020 and issues in scheduling appointments and collecting the blood samples, some patients had their last follow up visit at maximum 15 months. In addition, patients were advised to attend their follow up visits when they were able to safely attend the hospital. The impact of final $CD8^+$ T_{EMRA} sample collections at 15 months (rather than 12 months due to Covid-19) was explored using a linear model assuming Gaussian distribution and identity link.

3.11.7 Full Analysis Population

All analyses of primary and secondary outcomes were based on the full analysis population following the intention-to-treat principle. Hence, all patients were analysed according to their random allocation to either treatment or control group independent of whether they had received the treatment or not.

3.11.8 Per-Protocol Population

The Per-protocol population is defined as all subjects who took the drug continuously, and at the required dose until the final trial visit. Patients who stopped taking the drug for periods of time, or who stopped their trial medication early were excluded from the analysis. This will result in a smaller sample size and potential loss of statistical power and will only be considered for the primary outcome.

3.11.9 Subgroup analysis

Subgroup analysis on proportion of $CD8^+$ T_{EMRA} was conducted for type of ACS (STEMI or NSTEMI) and CMV (+ or -) at 12 months using linear mixed-effects models to estimate change from baseline between TA-65MD® vs placebo.

3.11.10 Missing Data

The proportion of missing data was tabulated in each arm. Analysis was performed both when missing data were ignored and when they were included. The impact of missing data was sensitised using multiple imputation techniques.

3.11.11 Methods to Minimise Bias

The trial statistician remained blinded to any randomisation and treatment group allocation until the end of data analysis to reduce accidental bias. The primary analyses and summary statistics were also reproduced using different models.

3.11.12 Treatment Compliance

At day 1 and at months 1, 3, 6, 9 and 12, study drug adherence was recorded by measuring Start date, Start dose time, End date, End dose time, Number of capsules dispensed, and Number of capsules returned as discrete variables and whether the patient took all doses on all days. Adherence and compliance to treatment at each visit were determined by whether a patient had taken at least 80% of their required doses during the trial.

3.11.13 Complier Average Causal Effects (CACE)

In randomised trials with imperfect compliance, the intention-to-treat (ITT) parameter may underestimate the effectiveness of an intervention because some patients, in either trial arm, may not adhere to their assigned treatment. The ITT principle provides the most robust estimate of the impact of the two treatments on change in proportion of $CD8^+$ T_{EMRA}, but it may not provide the actual difference between the two treatments when patients were adhered to less than 80% of their drug allocated doses post-randomisation. Complier average causal effect (CACE) analysis was performed to estimate the true impact of treatment on change in proportion of $CD8^+$ T_{EMRA}. CACE analysis allows unbiased assessment of intervention effect by weighting the intention-to-treat results by the proportion of compliers and non-compliers; where compliance is identified as taking at least 80% of allocated doses of TA-65MD.

3.11.14 Safety Analyses

The protocol mandated that the trial would stop if a patient died from a TA65-MD treatment-related event or if two patients experienced the same SUSAR with severe or life-threatening consequences. Safety data was not subject to statistical analysis but was summarised by arm and line listed at each severity category. For AEs occurring on multiple occasions, the rates of occurrence in each arm were reported.

The descriptive statistics of following variables were presented:

- AE
- AE severity
- AE causality
- Expectedness in relation to foreseeable AEs
- Suspected Unexpected Serious Adverse Reactions (SUSAR).

3.12 Summary of Changes to the Protocol

Duration of the trial and participation

The treatment period was extended due to the COVID-19 pandemic. Some patients continued the IMP for longer than 12 months to facilitate a final visit evaluation.

Patient Assessments

Due to the COVID-19 pandemic, patients were unable to attend hospital for their face-to-face follow up visits. The research team conducted AE assessment, changes to concomitant medications and IMP compliance assessment over the telephone. However, blood samples, endothelial function assessment and echocardiogram that were due to occur at the 6- and 12-month visits were carried out when the patients were able to safely attend hospital.

Receiving IMP

Patients were unable to attend site for face-to-face visits at some points during the COVID-19 pandemic, Instead, IMP was delivered to patients. The site team also dispensed additional kits to ensure a continuous supply of IMP.

Statistical Analysis

Primary outcome of immunosenescence at 1 year was assessed at a later timepoint for some patients due to COVID-19 pandemic.

Consent

Patients provided additional verbal consent to their continuation in the trial beyond 12 months.

Chapter Four
Results

4.1 Trial progress

Site initiation visit was performed on 11/1/2019. Patient recruitment started 21/1/2019 and was completed 17/3/2020 (**Figure 8**). The final trial visit was 18/3/2021. Of 310 patients assessed for eligibility, 90 patients were randomised to placebo or TA-65 and 81 patients completed primary outcome sample analyses (**Figure 9**). No patients were lost to follow up; 15 patients (7 from placebo group and 8 from TA-65) discontinued trial medications (**Table 9**) and of those, 7 attended for primary outcome sample analyses. Due to COVID-19 related trial visit restrictions, 20 patients (10 in each group) continued trial medication for 15 months to allow measurement of final endpoints. Endpoint analyses were completed on 22/7/2021.

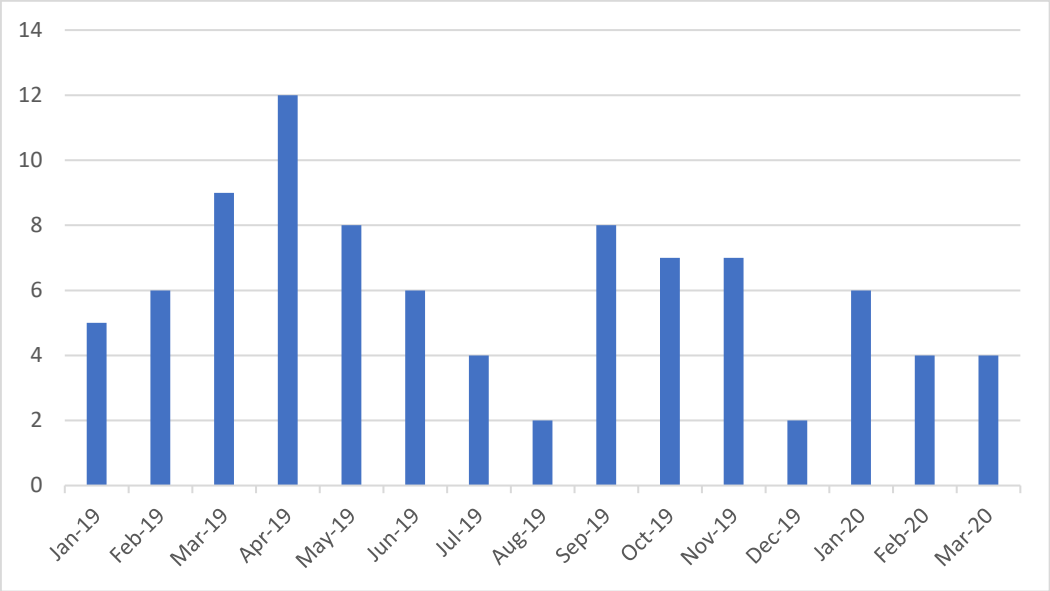

Figure 8: Recruitment progress

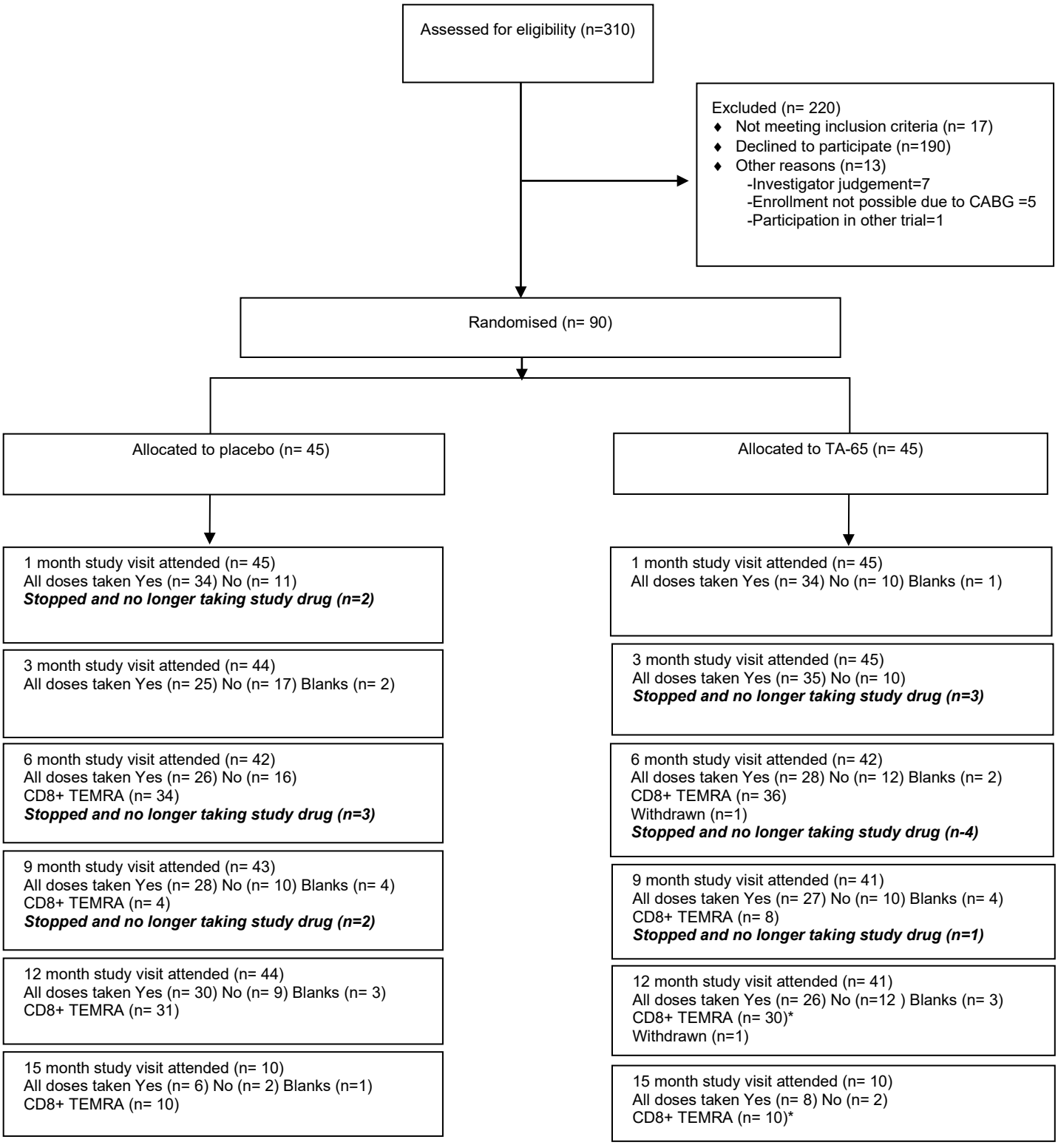

Figure 9. Trial flow diagram

Reasons for stopping IMP	Notes
Patient decision AE– 5 Personal choice – 5 Other- 1	AE included joint pain, chest infection, pulmonary embolism, nausea, proctitis
Principal investigator decision Cancer diagnosis – 3	Two patients had undiagnosed cancer at time of randomisation
IDMEC decision Inclusion criteria not met – 1	Known but undeclared low grade prostate cancer at baseline

Table 9: Reasons for stopping IMP before schedule. IMP= Investigational Medicinal Product; AE= adverse event; IDMEC = independent data monitoring and events committee.

4.2 Baseline characteristics

The median age of patients who underwent randomization was 71 years, 16.7% were women, 72.2 % were CMV seropositive (**Table 10**). All participants were of white ethnicity. The placebo group included more smokers or ex-smokers (82.2% vs. 62.2%), hyperlipidaemia (73.3% vs. 55.6%) and more previous MI (24.4% vs. 11.1%). There was a high usage of evidence-based ACS drug treatment in both groups.

	Placebo (N=45)	**TA-65 (N=45)**
Baseline characteristics		
Age, median, y (IQR)	71.4 (6.7)	70.9 (6.6)
Male sex, n (%)	38 (84.4)	37 (82.2)
BMI, mean (SD)	27.3 (4.2)	28.4 (4.8)
Family history of IHD, n (%)	19 (42.2)	20 (44.4)
Current or ex-smoker, n (%)	37 (82.2)	28 (62.2)
Past medical history, n (%)		
Atrial fibrillation or flutter	6 (13.3)	1 (2.2)
Hypertension	21 (46.7)	24 (53.3)
Hyperlipidaemia	33 (73.3)	25 (55.6)
Type 2 diabetes mellitus	5 (11.1)	8 (17.8)
Previous MI	11 (24.4)	5 (11.1)
Previous PCI	6 (13.3)	7 (15.6)
Previous CABG	6 (13.3)	2 (4.4)
PVD	2 (4.4)	4 (8.9)
Previous TIA or CVA	5 (11.1)	2 (4.4)
COPD or asthma	6 (13.3)	7 (15.6)
CMV seropositive	34 (75.6)	31 (68.9)
Baseline medications, n(%)		
Aspirin	45 (100)	45 (100)
P2Y12 inhibitors	44 (98)	45 (100)
Statins	44 (98)	45 (100)
ACE-inhibitors/ARB	43 (96)	43 (96)
Clinical measurements, mean (SD)		
Systolic BP, mmHg	119 (17)	123 (17)
Diastolic BP, mmHg	72 (11)	72 (10)
Glucose, mmol/L	6.8 (1.5)	7.1 (2.3)
Total cholesterol, mmol/L	4.5 (1.2)	4.4 (1.3)
LDL-C, mmol/L	2.5 (1.2)	2.6 (1.1)
HDL-C, mmol/L	1.3 (0.7)	1.1 (0.3)

Table 10. Comparison of baseline characteristics between placebo and TA-65 groups.

4.3 ACS characteristics

There were 47 (52.2%) NSTEMIs and 43 (47.8%) STEMIs with a predominance of single vessel disease (53.3%). Most patients (86.7%) underwent PCI.

	Placebo (N=45)	**TA-65 (N=45)**
ACS type, n(%)		
NSTEMI	23 (51.1)	24 (53.3)
STEMI	22 (48.9)	21 (46.7)
Presenting ECG		
ST elevation/LBBB	24 (53.3)	24 (53.3)
ST depression	3 (6.7)	1 (2.2)
T wave inversion	7 (15.6)	10 (22.2)
No acute	11 (24.4)	10 (22.2)
Infarct location, n (%)		
Anterior	14 (31.1)	14 (31.1)
Non anterior	27 (60.0)	23 (51.1)
Unknown	4 (8.9)	8 (17.8)
No. of vessel disease, n (%)		
One	26 (57.8)	22 (48.9)
Two	16 (35.6)	15 (33.3)
Three	3 (6.7)	8 (17.8)
ACS treatment, n (%)		
PCI	41 (91.1)	37 (82.2)
Medical	4 (8.9)	6 (13.3)
CABG	0 (0)	2 (4.4)

Table 11. Comparison of ACS characteristics between placebo and TA-65 groups.

4.4 PCI characteristics

PCI was performed in 41 and 37 patients in the placebo and TA-65 groups respectively. Most PCI were performed via the radial access (97%). Number of lesions attempted were similar in both groups. Median number of stents were higher in the TA-65 group (2 vs 1).

	Placebo (N=41)	**TA-65 (N=37)**
No. of vessels attempted, median (IQR)	1 (0)	1 (0)
Vessel attempted LMS Proximal LAD Other LAD LC RCA Vein graft	 2 (4.9) 4 (9.7) 9 (21.9) 11 (26.8) 13 (31.7) 2 (4.9)	 2 (5.4) 8 (21.6) 6 (16.2) 4 (10.8) 15 (40.5) 2 (5.4)
No. of lesions attempted, median (IQR)	1 (0)	1 (0)
No. of drug eluting stents, median (IQR)	1 (1)	2 (1)
Maximum target vessel diameter, mean (SD), mm	3.6 (0.7)	3.7 (0.6)
Total stent length, mean (SD), mm	36.7 (22.3)	43.3 (23.7)

Table 12: Procedure details for percutaneous coronary intervention performed in TA-65 and placebo groups. LMS= Left main stem, LAD= Left anterior descending artery, LC= Left circumflex, RCA= Right coronary artery

4.5 Effect on primary endpoint

4.5.1 Baseline percentage CD8+ TEMRA

There was a high percentage of $CD8^+$ T_{EMRA} (>45%) in both groups at baseline (77.8%) (**Figure 10**). This reflects the expected high burden of senescent cells in post ACS elderly patients.

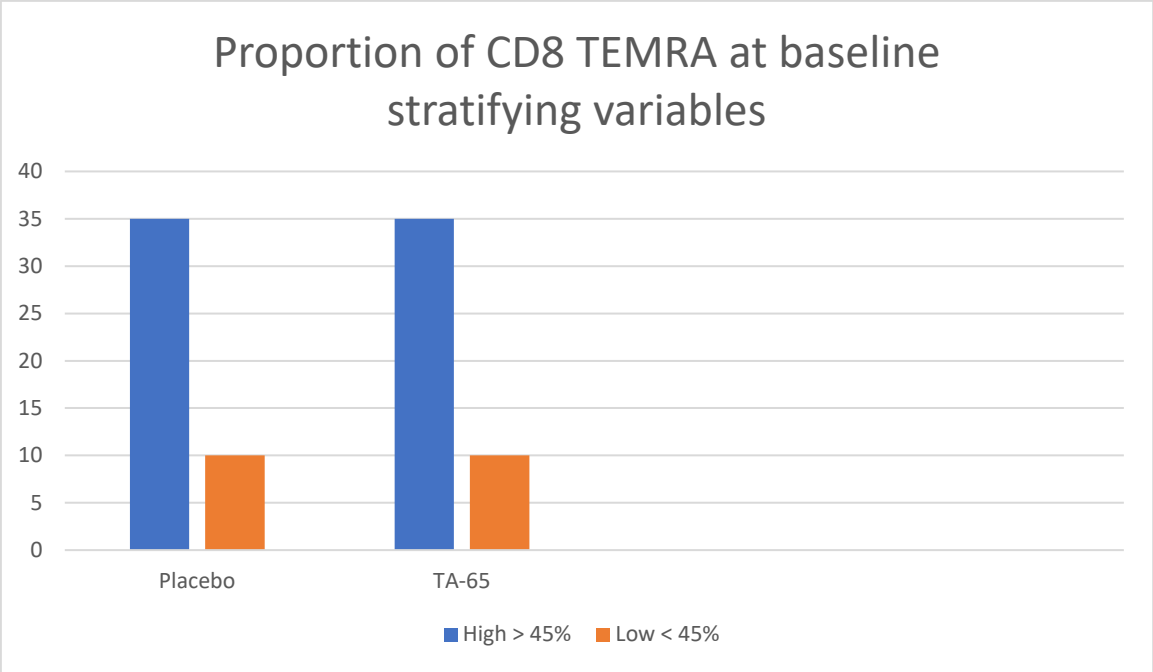

Figure 10: Bar plot for $CD8^+$ T_{EMRA} baseline stratifying variable within trial arms.

4.5.2 Primary analysis

Percent $CD8^+$ T_{EMRA}									
Time Points	Placebo			TA-65			Treatment effect		
	n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	B-A: Change from Baseline Diff(95% CI)
Baseline	45	59.1(19.31)		45	58.1(21.66)		90	58.6(20.41)	
12 months	41	61.01(19.77)	2.2(-0.13,4.52)	40	60.85(21.02)	2.03(-1.36,5.51)	81	60.93(20.27)	-0.2(-4.25,3.89)

Table 13. Treatment effect on the primary endpoint in placebo and TA-65 groups.

The treatment effect on the primary outcome was estimated as -0.2, showing that TA-65 treatment decreased the percentage of $CD8^+$ T_{EMRA} as compared to placebo from baseline to 12 month, however it was not a significant effect. There were no significant differences in the percentage of $CD8^+$ T_{EMRA} in both groups at 12 months compared to baseline.

4.5.3 Effect of minimisation factors

Variable	Effect estimate	95% CI
Gender (Male vs Female)	2.90	(-5.69,11.49)
ASC type (STEMI vs NSTEMI)	-3.42	(-9.78,2.93)
$CD8^+$ T_{EMRA} Baseline (low ≤45 vs high > 45%)	-30.21	(-37.79, -22.64)

Table 14. Effect estimate of minimisation factors.

Gender and ACS type did not significantly affect the outcome. A low baseline $CD8^+$ T_{EMRA} decreased the outcome compared to high baseline $CD8^+$ T_{EMRA}.

4.5.4 Analysis considering 3 time points

Percent $CD8^+$ T_{EMRA}									
Time Points	Placebo			TA-65			Treatment effect		
	n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	Change from Baseline Diff(95% CI)
Baseline	45	59.1(19.31)		45	58.1(21.66)		90	-1(29.53)	
6 months	36	58.97(18.52)	1.59(-0.58,3.73)	37	64.26(18.66)	2.67(-0.55,6)	73	5.82(25.07)	1.07(-2.79,5)
12 months	41	61.01(19.77)	2.19(0.14,4.24)	40	60.85(21.02)	1.85(-1.3,5.05)	81	-1.18(29.49)	-0.35(-4.08,3.4)

Table 15. Treatment effect on the primary endpoint in placebo and TA-65 groups.

The power of the hypothesis testing was increased by analysing 3 time points. In the placebo group, the mean percentage of $CD8^+$ T_{EMRA} significantly increased by 2.19 from baseline to 12 months. The treatment effect was still a non-significant effect from baseline to 12 months.

4.5.5 Subgroup analysis

Percent $CD8^+$ T_{EMRA}										
Sub Groups	**Time Points**	**Arm A**			**Arm B**			**Total**		
		n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	B-A: Change from Baseline Diff(95% CI)
ACS(STEMI)	Baseline	22	58.27(17.69)		21	54.23(21.68)		43	56.29(19.61)	
	6 month	17	61.25(18.84)	2.26(-1.02,5.54)	18	61.84(18.75)	4.53(0.57,8.63)	35	61.55(18.52)	2.31(-2.79,7.49)
	12 month	20	61.59(18.85)	2.93(-0.16,6.02)	20	59.89(19.87)	4.32(0.49,8.23)	40	60.74(19.14)	1.4(-3.46,6.31)
		n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	B-A: Change from Baseline Diff(95% CI)
ACS(NSTEMI)	Baseline	23	59.9(21.1)		24	61.5(21.52)		47	60.72(21.1)	
	6 month	19	56.93(18.5)	1.02(-1.89,3.88)	19	66.55(18.79)	0.91(-4.06,6.06)	38	61.74(19.03)	-0.1(-5.79,5.69)
	12 month	21	60.45(21.06)	1.52(-1.26,4.29)	20	61.81(22.58)	-0.57(-5.5,4.4)	41	61.12(21.55)	-2.09(-7.64,3.49)

Table 16. Treatment effect on the primary endpoint in placebo and TA-65 groups stratified to ACS type.

		n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	B-A: Change from Baseline Diff(95% CI)
CMV(Positive)	Baseline	34	64.88(16.9)		31	64.47(19.93)		65	64.69(18.26)	
	6 month	28	64.21(15.87)	1.51(-0.75,3.75)	26	69.64(16.85)	0.32(-3.35,4.19)	54	66.82(16.42)	-1.2(-5.42,3.1)
	12 month	31	66.77(15.88)	2.09(-0.07,4.26)	28	64.62(21.63)	-0.28(-3.9,3.41)	59	65.75(18.69)	-2.39(-6.49,1.74)
		n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	B-A: Change from Baseline Diff(95% CI)
CMV(Negative)	Baseline	11	41.23(15.26)		14	44(18.99)		25	42.78(17.16)	
	6 month	8	40.63(15.81)	1.9(-4.08,7.62)	11	51.55(17.05)	7.84(1.97,13.83)	19	46.95(17.01)	6.17(-2.05,14.62)
	12 month	10	43.13(20.67)	2.6(-2.86,7.87)	12	52.06(17.26)	6.77(1.14,12.65)	22	48(18.97)	4.21(-3.53,12.14)

Table 17. Treatment effect on the primary endpoint in placebo and TA-65 groups stratified to CMV status.

STEMI or NSTEMI did not affect level of $CD8^+$ T_{EMRA} at baseline. The treatment effect on the outcome was not significant in both groups. CMV positive patients had higher levels of baseline $CD8^+$ T_{EMRA}. However, CMV status did not affect the treatment effect.

4.5.6 Complier average casual effect analysis

CACE analysis assessed the treatment effect among the patients who are known as compliers. The treatment effect of TA-65 on the primary outcome was estimated as -0.2 from baseline to 12 months, however this was not a significant effect. In other words, the treatment effect stays the same over this period.

Percent $CD8^+$ T_{EMRA}						
Time Points	**Placebo**		TA-65		**Total**	
	Complier n/N(%)	CACE Change from Baseline Diff(95% CI)	Complier n/N(%)	CACE Change from Baseline Diff(95% CI)	Complier n/N(%)	CACE Change from Baseline Diff(95% CI)
Baseline	39/39(100)		37/37(100)		76/76(100)	
12 months	37/37(100)	2.2(-0.13,4.52)	37/37(100)	2.03(-1.36,5.51)	74/74(100)	-0.2(-4.25,3.89)

Table 18. CACE analysis of treatment effect on primary outcome

4.5.7 Effect of missing data

The plot of missing CD8$^+$ T_{EMRA} data is presented in **Figure 11**. For the analysis, a 2-stage multiple imputation with 100 imputations has been done to estimate the missing values (**Table 19**).

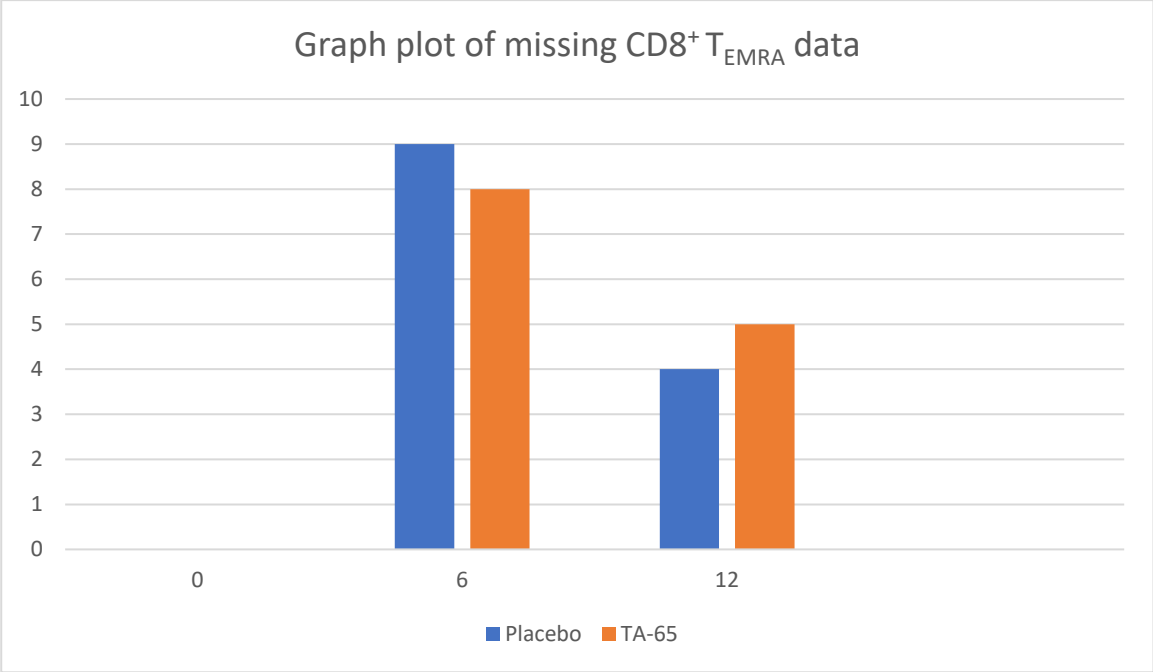

Figure 11. Bar plot of number of missing $CD8^+$ T_{EMRA} values at each visit

Percent $CD8^+$ T_{EMRA}									
Time Points	**Placebo**			TA-65			Treatment effect		
	n	Mean(SD)	Change from Baseline Diff(P-value)	n	Mean(SD)	Change from Baseline Diff(P-value)	n	Mean(SD)	Change from Baseline Diff(P-value)
Baseline	45	59.1(19.31)		45	58.1(21.66)		90	-1(2.35)	
6 months	45	60.7(18.94)	1.6(0.16)	45	60.77(19.67)	2.66(0.11)	90	-0.11(0.73)	0.89(0.65)
12 months	45	61.25(19.23)	2.15(0.04)	45	60.01(21.34)	1.9(0.24)	90	-1.33(2.03)	-0.33(0.86)

Table 19. Summary of sensitivity analysis on percentage $CD8^+$ T_{EMRA} using multiple imputation

4.6 Effect on T subpopulations

4.6.1 Effect on % $CD8^+$ and $CD4^+$ cell subtypes

In both groups, $CD8^+$ T cells consist predominantly of senescent $CD8^+$ T_{EMRA} while the majority of CD4 T cells are naïve and central memory cells. There were no significant changes at 12 months in all % $CD8^+$ and $CD4^+$ T cell subsets (**Table 20**).

T-lymphocyte subset, % of parent population (SD)	**Placebo**			**TA-65**		
	Baseline N=45	**6 months N=36**	**12 months N=41**	**Baseline N=45**	**6 months N=37**	**12 months N=40**
$CD8^+$	24.1(14.3)	20.9(11.4)	20.1(10.2)	26.9(15.2)	26.1(16.4)	24.4(14.0)
$CD8^+$ T_N	7.23(6.1)	5.88(4.31)	5.95(4.02)	6.88(5.08)	6.44(4.51)	6.17(4.45)
$CD8^+$ T_{CM}	11.54(7.5)	11.3(7.0)	11.1(7.8)	12.1(9.8)	10.4(7.7)	11.0(8.4)
$CD8^+$ T_{EM}	22.1(11.8)	23.8(12.0)	22.0(12.0)	22.9(15.2)	18.9(11.5)	21.9(16.0)
$CD8^+$ T_{EMRA}	59.1 (19.3)	59.0 (18.5)	61.0 (19.8)	58.1 (21.7)	64.3 (18.7)	60.8 (21.0)
$CD4^+$	64.3(14.7)	66.5(12.7)	65.98(12.7)	61.06(14.8)	59.8(16.2)	61.9(15.1)
$CD4^+$ T_N	42.3(14.3)	45.25(15.0)	46.95(14.2)	40(16.0)	41.8(15.0)	44.6(15.5)
$CD4^+$ T_{CM}	42.7(13.0)	41.5(13.0)	39.8(13.9)	42.4(14.6)	42.8(14.1)	41.3(13.9)
$CD4^+$ T_{CM}	12.3(6.9)	10.9(6.4)	10.6(5.9)	14(13.7)	11.73(9.5)	10.5(9.0)
$CD4^+$ T_{EMRA}	2.6(3.8)	2.3(3.3)	2.6(4.3)	3.6(6.7)	3.6(7.3)	3.61(8.1)

Table 20. Raw data showing the proportion of $CD4^+$ and $CD8^+$ T-lymphocytes subsets (% of parent population) identified by flow cytometry. Results shown as the mean value for each treatment arm at each time point.

4.6.2 Effect on lymphocytes

In the placebo group, the mean total lymphocyte count changed from 1686 to 1640 cells/µL (modelled as -45, 95% CI: -150 – 60) over 12 months. In the TA-65 group, this increased significantly from 1666 to 1914 cells/µL (modelled as +240, 95% CI: 109 – 374) (**Table 21, Figure 13**). The treatment effect at 12 months compared to baseline was estimated as +285 cells/µL (95% CI: 117 – 452). This was due to increases of similar magnitude in all major populations of lymphocytes in the TA-65 group: total T-lymphocytes (+14.0%), CD4+ (+14.0%) and CD8+ T-lymphocytes (+13.7%), B-lymphocytes (+14.3%) and natural killer cells (+15.9%) (**Figure 12**).

Cell type, cells/µL (SD)	**Placebo**			**TA-65**		
	Baseline N=45	**6 months N=36**	**12 months N=42**	**Baseline N=45**	**6 months N=37**	**12 months N=40**
Lymphocytes	1686.0 (591.2)	1609.1 (543.8)	1640.6 (596.1)	1666.3 (594.1)	1762.9 (495.4)	1914 (571.8)
T-lymphocytes: $CD3^+$	1270.7 (488.3)	1212.3 (465.6)	1241.5 (504.1)	1208.8 (462.0)	1307.0 (405.1)	1392.8 (444.0)
$CD3^+CD4^+CD8^-$	801.9 (345.4)	800.9 (333.7)	811.2 (361.9)	731.2 (291.9)	769.5 (269.9)	831.9 (284.6)
$CD3^+CD4^+CD8^-CD28^-$	53.5 (101.8)	41.8 (55.1)	48.5 (76.4)	37.2 (59.3)	46.0 (67.4)	50.6 (84.5)
$CD3^+CD4^-CD8^+$	391.1 (286.7)	339.4 (253.8)	355.0 (236.4)	396.7 (263.0)	442.9 (290.5)	472.9 (292.1)
$CD3^+CD4^-CD8^+CD28^-$	64.8 (19.57)	64.9 (19.08)	65.81 (19.74)	58.16 (22.83)	64.87 (18.51)	64.02 (21.09)
NK cells: $CD3^-CD16^+CD56^+$	245.3 (129.5)	217.6 (101.7)	227.3 (122.8)	288.2 (174.5)	286.4 (157.6)	324.0 (201.3)
B-lymphocytes: $CD3^-CD19^+$	155.2 (93.3)	163 (93.9)	157.2 (86.5)	155.2 (103.1)	155.3 (91.6)	181.1 (121.4)

Table 21. Raw data showing the absolute counts of adaptive immune cells as measured by TruCount flow cytometry. Results shown as the mean value for each treatment arm at each time point.

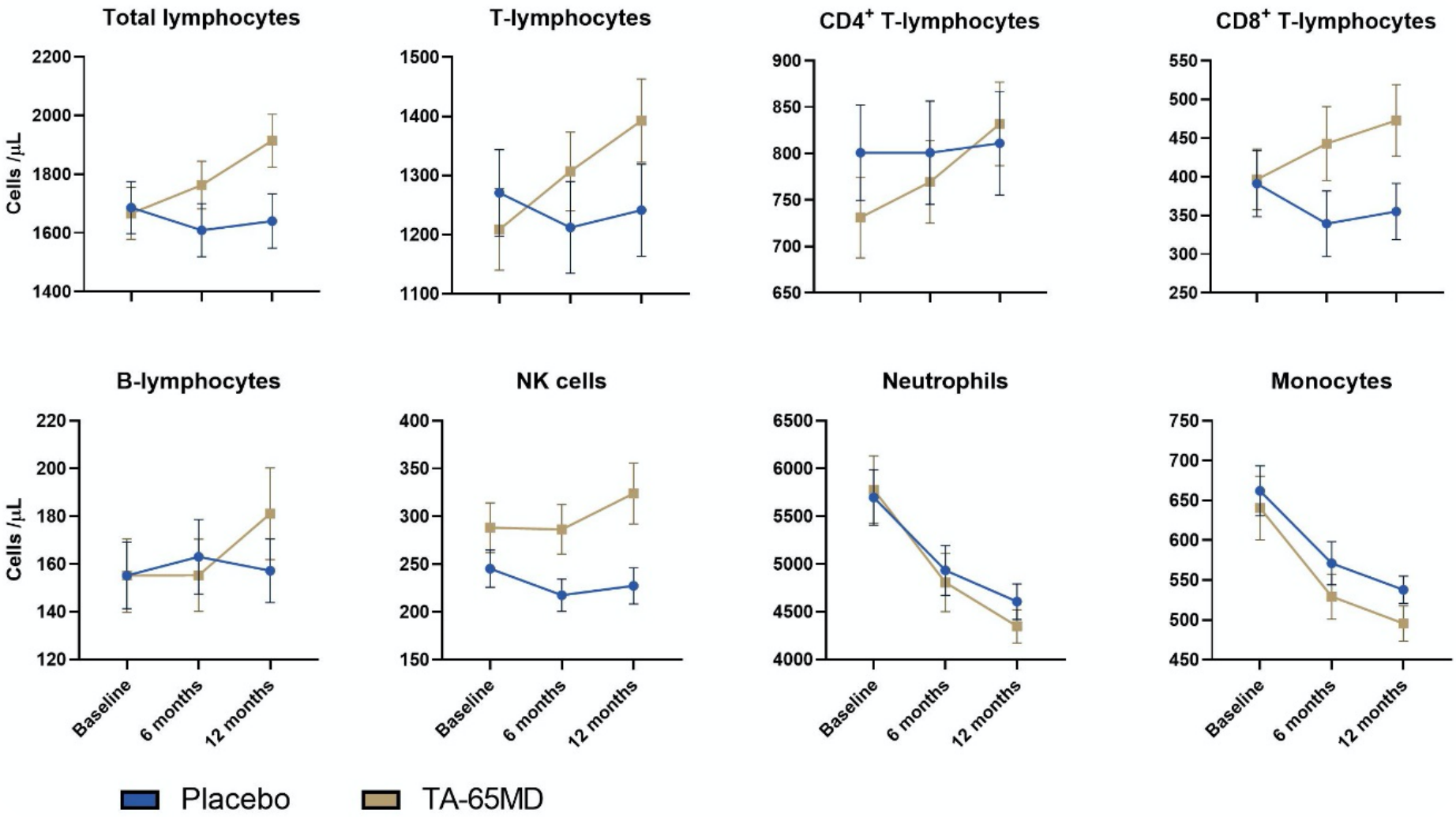
Total lymphocytes
T-lymphocytes
CD4+ T-lymphocytes
CD8+ T-lymphocytes
B-lymphocytes
NK cells
Neutrophils
Monocytes
Cells /µL
2200
2000
1800
1600
1400
1500
1400
1300
1200
1100
900
850
800
750
700
650
550
500
450
400
350
300
250
220
200
180
160
140
120
400
350
300
250
200
150
6500
6000
5500
5000
4500
4000
750
700
650
600
550
500
450
Baseline
6 months
12 months
Placebo
TA-65MD

Figure 12 - The proportionate change in mean cell counts at each visit compared to baseline per treatment arm. The change in mean cell counts at 6 and 12 months, as a proportion of mean value at baseline for each treatment arm.

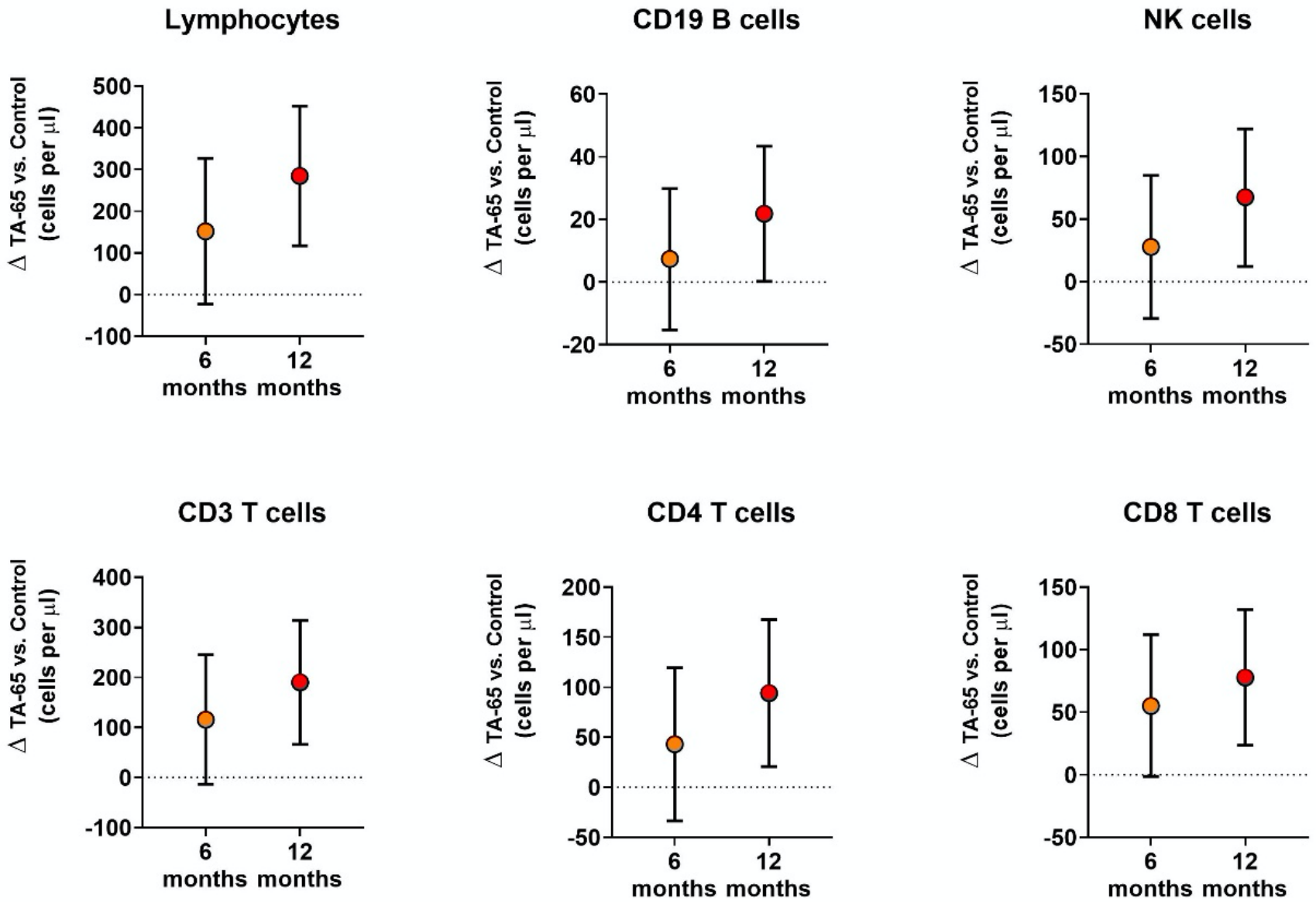
Lymphocytes
Δ TA-65 vs. Control (cells per μl)
500
400
300
200
100
0
-100
6 months
12 months
CD19 B cells
Δ TA-65 vs. Control (cells per μl)
60
40
20
0
-20
6 months
12 months
NK cells
Δ TA-65 vs. Control (cells per μl)
150
100
50
0
-50
6 months
12 months
CD3 T cells
Δ TA-65 vs. Control (cells per μl)
400
300
200
100
0
-100
6 months
12 months
CD4 T cells
Δ TA-65 vs. Control (cells per μl)
200
150
100
50
0
-50
6 months
12 months
CD8 T cells
Δ TA-65 vs. Control (cells per μl)
150
100
50
0
-50
6 months
12 months

Figure 13. Treatment effect of TA-65MD on absolute counts of lymphocytes after 6 and 12 months of treatment. The estimated change in the outcomes and their associated 95% confidence interval among the patients treated with TA-65MD compared to Placebo at 6 month and 12 months compared to baseline. If for an outcome, after a particular visit, the associated 95% CI does not include zero, it shows the estimated treatment effect has significantly changed over the time.

4.6.3 Effect on innate immunity

In contrast to lymphocytes, neutrophil granulocytes, classical as well as non-classical monocytes were reduced after 6 and 12 months compared to baseline (**Table 22**) but did not differ significantly at 12 months between treatment arms (**Figure 14**).

Cell type, cells/μL (SD)	**Placebo**			**TA-65**		
	Baseline N=45	**6 months N=36**	**12 months N=42**	**Baseline N=45**	**6 months N=37**	**12 months N=40**
Monocytes: $CD3^{-}CD4^{+}CD19^{-}CD45^{+}$	662.1 (209.4)	571.2 (162.5)	537.8 (111.6)	640.6 (267.5)	529.3 (171.1)	495.5 (141.5)
Classical: $CD3^{-}CD4^{+}CD19^{-}CD45^{+}CD16^{low}$	532.0 (177.9)	460.9 (149.5)	442.0 (99.7)	523.3 (230.5)	462.7 (221.1)	407.3 (111.6)
Non-classical: $CD3^{-}CD4^{+}CD19^{-}CD45^{+}CD16^{high}$	25.5 (23.5)	17.7 (27.6)	14.4 (21.6)	22.2 (27.6)	19.68 (24.3)	14.62 (20.7)
Intermediate: $CD3^{-}CD4^{+}CD19^{-}CD45^{+}CD16^{int}$	89.9 (48.7)	83.8 (47.8)	76.0 (44.4)	82.78 (48.8)	87.05 (59.9)	75.83 (47.6)
Neutrophils	5696.5 (1941.4)	4933.8 (1566.6)	4606.8 (1209.2)	5778.9 (2365.1)	4806.6 (1856.3)	4348.7 (1101.4)

Table 22. Raw data showing the absolute counts of innate immune cells as measured by TruCount flow cytometry. Results shown as the mean value for each treatment arm at each time point.

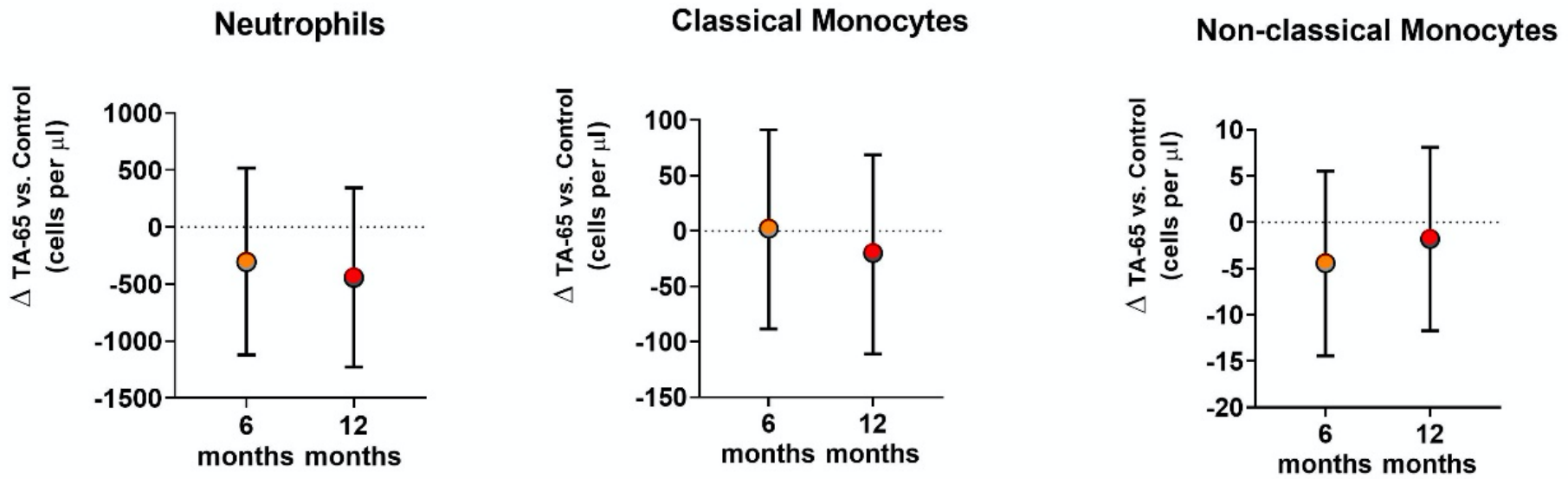

Figure 14. Treatment effect of TA-65MD on absolute counts of neutrophils and monocytes after 6 and 12 months of treatment.

4.7 Effect on high-sensitive C-reactive protein

High-sensitive C-reactive protein levels were similar in TA-65MD and placebo groups at baseline (11.9 vs 10.9 mg/L). After 12 months of treatment, hsCRP levels in patients taking TA-65MD were 62.1% lower than in those taking placebo (1.1 vs 2.9 mg/L). The reduction in hsCRP during 12 months of treatment was estimated to be 2.95 mg/L greater in the TA-65MD arm (95% CI for treatment effect: -12.11 – 6.21).

STEMI patients had higher baseline levels of hsCRP (18.1 and 17.9 mg/L for placebo and TA-65, respectively) than NSTEMI patients (4.4 and 6.7 mg/L). In STEMI patients, the mean hsCRP value at 12 months was 71.8% lower in the TA-65MD group compared to placebo (1.1 vs 3.9mg/L); in NSTEMI patients, this was 45.0% lower (1.1 vs 2.0 mg/L) (**Table 23, Figure 15**).

We also observed an interesting trend in the TA-65 group, where each increase in lymphocyte count of 100 cells per microliter, or 0.1 $x10^9$/mL, correlated with a 0.95 mg/L greater decrease in hsCRP between baseline and 12 months, although this correlation was not statistically significant (95% CI: -2.28 – 0.42) (**Table 24**).

High-sensitivity CRP										
ACS subtype	**Time Points**	Placebo			TA-65			Treatment effect		
		n	Mean (SD)	Change from Baseline Diff (95% CI)	n	Mean (SD)	Change from Baseline Diff (95% CI)	n	Mean (SD)	Change from Baseline Diff (95% CI)
STEMI	Baseline	20	18.1 (33.0)	-	20	17.9 (34.7)	-	40	18.0 (33.4)	-
	6 months	17	5.6 (14.1)	-11.2 (-24.5, 2.1)	17	1.9 (3.3)	-16.2 (-29.3,-3.1)	34	3.8 (10.3)	-4.2 (-23.0, 14.5)
	12 months	20	3.9 (9.0)	-13.4 (-26.1, -0.7)	20	1.1 (1.0)	-16.8 (-29.3,-4.2)	40	2.5 (6.5)	-3.0 (-20.9, 15.0)
NSTEMI	Baseline	22	4.4 (6.4)	-	23	6.7(12.7)	-	45	5.6 (10.1)	-
	6 months	19	1.4 (1.4)	-3.0 (-5.5, -0.6)	19	1.5 (1.4)	-5.3 (-9.9,-0.6)	38	1.4 (1.4)	-2.3 (-7.5, 3.0)
	12 months	22	2.0 (2.6)	-2.5 (-4.8, -0.1)	20	1.1 (0.9)	-5.6 (-10.2,-0.9)	42	1.6 (2.0)	-3.1 (-8.2, 2.0)

Table 23. Effect of TA-65 on hsCRP stratified to ACS subtype.

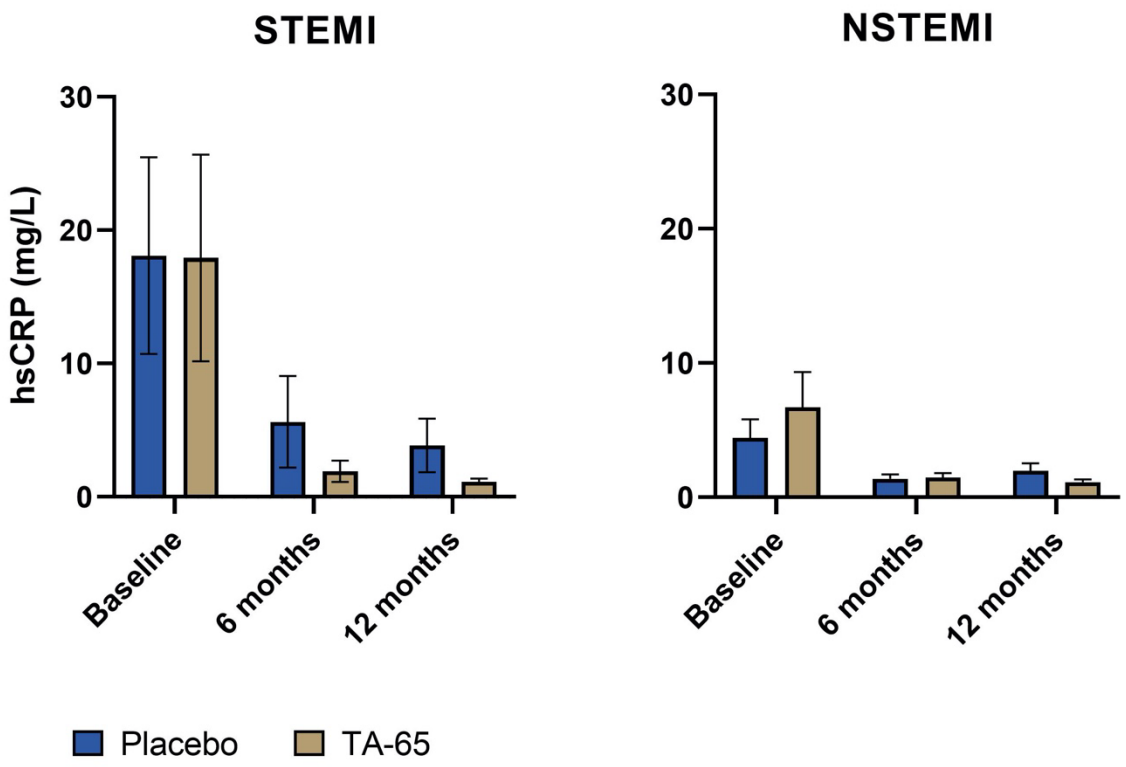

Figure 15. Mean hsCRP values at baseline, 6 months and 12 months in each treatment group, split by MI type. STEMI = ST-elevation myocardial infarction. NSTEMI = non-ST elevation myocardial infarction.

High-sensitivity CRP								
Time Points	**Placebo**			**TA-65**			**Treatment effect**	
	n	Mean(SD)	Change from Baseline for one unit increase in Lymphocytes (95% CI)	n	Mean(SD)	Change from Baseline for one unit increase in Lymphocytes (95% CI)	n	Change from Baseline for one unit increase in Lymphocytes Diff(95% CI)
Baseline	42	10.93 (23.92)	-	43	11.92 (25.69)	-	85	-
12 months	42	2.87 (6.46)	0.0001 (-0.0118 – 0.012)	40	1.13 (0.94)	-0.0095 (-0.023 – 0.004)	82	-0.009 (-0.027 – 0.009)

Table 24. Effect of lymphocyte count on change in CRP between baseline and 12 months. In the placebo group, increased lymphocyte count was not associated with reduction in CRP. In the TA-65 group, a one unit (cell/microliter) increase in total lymphocyte count was associated with a 0.0095mg/L reduction in hsCRP, although this was not statistically significant.

4.8 Effect on endothelial function

Mean baseline RHI was 1.85. Eight (9%) patients had RHI < 1.35 considered to be highly sensitive and specific for coronary endothelial dysfunction. The treatment effect on RHI is estimated as 0.06 from baseline to 6 months and 0.03 from baseline to 12 months (**Table 25**). However, the change is not statistically significant. Moreover, there are no significant changes at 6 and 12 months in both groups.

Endothelial Function (RHI)									
Time Points	Placebo			TA-65			Treatment effect		
	n	Mean (SD)	Change from Baseline Diff (95% CI)	n	Mean (SD)	Change from Baseline Diff (95% CI)	n	Mean (SD)	Change from Baseline Diff (95% CI)
Baseline	45	1.85 (0.51)	-	44	1.93 (0.53)	-	89	0.08 (0.56)	-
6 months	35	1.73 (0.49)	-0.1 (-0.31,0.09)	37	1.87 (0.35)	-0.03 (-0.22,0.15)	72	0.13 (0.63)	0.06 (-0.21,0.33)
12 months	37	1.82 (0.51)	-0.03 (-0.22,0.17)	39	1.95 (0.64)	0 (-0.18,0.19)	76	0.14 (0.86)	0.03 (-0.23,0.3)

Table 25. Endothelial function, as measured by reactive hyperaemia index (RHI), in placebo and TA-65 arms at baseline, 6 months and 12 months.

4.9 Effect on telomerase activity

Telomerase activity did not change significantly from baseline to 12 months in either group. The estimated change in telomerase activity was +22.9 (95% CI -91.8 – 136.0) in the placebo group, and +18.5 (-89.4 – 125.9) in the TA-65MD group (**Table 26**). Overall, there was no treatment effect with TA-65 (-4.82, 95% CI: -160.2 – 151.08).

Time Points	Placebo			TA-65			Treatment effect		
	n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	Change from Baseline Diff(95% CI)
Baseline	41	540.56(378.89)		42	524.76(306.13)		83	-39.87(401.2)	
6 months	36	559.39(332.15)	12.02(-97.33,122.06)	36	538.19(347.23)	27.07(-80.13,131.41)	72	-36.12(365.77)	14.55(-137.68,165.09)
12 months	33	552.15(269.04)	22.91(-91.82,136.01)	34	541.71(283.3)	18.46(-89.44,125.94)	67	-49.03(353.25)	-4.82(-160.2,151.08)

Table 26. Telomerase activity in placebo and TA-65 arms at baseline, 6 months and 12 months.

4.10 Effect on NT proBNP

There was a reduction of NT-proBNP from 988 to 299pg/mL after 12 months (69.7% reduction) in the TA-65 group (**Table 27**). A smaller reduction of 45.1% was seen in the placebo group (947pg/mL to 520pg/mL). However, the treatment effect of TA-65 after 12 months was not statistically significant (-303, 95% CI: -746 – 142). Analysis by ACS type and CMV status showed no significant change in treatment effect.

NT proBNP									
Time Points	Placebo			TA-65			Treatment effect		
	n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	Change from Baseline Diff(95% CI)
Baseline	45	947.4(1084.47)		45	988.09(1408.65)		90	40.69(1813.84)	
6 months	36	262.08(302.74)	-579.08(-903.28,-263.37)	36	277.14(328.78)	-738.55(-1065.68,-403.57)	72	15.28(470)	-159.02(-618.97,310.78)
12 months	42	519.95(1439.06)	-421.88(-724,-118.76)	40	299.08(348.82)	-713.68(-1031.87,-391.71)	82	31.54(445.24)	-302.94(-746.2,141.8)

Table 27. NT proBNP levels in placebo and TA-65 arms at baseline, 6 months and 12 months.

4.11 Effect on echocardiographic parameters

Treatment with TA-65 also did not produce significant effects on change in LV end diastolic volume (treatment effect estimated as +0mL, 95% CI: -0.3 – 0.3), LV ejection fraction (-2.4, 95% CI -6.3 – 1.6) and peak systolic global longitudinal strain (-0.1, 95% CI: -2.3 – 2.0) at 12 months (**Table 28**).

	Placebo		**TA-65**		**Treatment effect**
Mean (SD)	**Baseline N=44**	**12 months N=39**	**Baseline N=44**	**12 months N=36**	
LV end systolic dimension (M-mode), cm	3.4 (0.7)	3.5 (0.8)	3.1 (0.8)	3.3 (0.7)	0.1 (-0.2, 0.5)
LV end diastolic dimension (M-mode), cm	4.7 (0.5)	4.7 (0.6)	4.6 (0.5)	4.6 (0.7)	0.0 (-0.3, 0.3)
LV end systolic volume, mL	39.2 (15.7)	39.9 (18.8)	38.4 (18.0)	38.2 (15.6)	3.7 (-2.8,9.8)
LV end diastolic volume, mL	86.2 (21.8)	89.0 (24.2)	86.4 (24.2)	83.8 (28.8)	-1.4 (-13.1,9.6)
LV ejection fraction, %	52.2 (11.4)	54.2 (11.2)	55.7 (10.7)	56.0 (7.6)	-2.4 (-6.3,1.6)
Peak systolic global longitudinal strain, %	76	-16.4 (4.0)	-16.5 (7.4)	-18.4 (4.2)	-0.1 (-2.3, 2.0)

Table 28. Echocardiographic parameters in placebo and TA-65 arms at baseline and 12 months.

4.12 Effect on oxidative stress

Treatment with TA-65 also did not impact on oxidative stress (estimated treatment effect: +0.08, 95% CI: -0.11 – 0.26) (**Table 29**).

Ox_TBARS									
Time Points	Placebo			TA-65			Treatment effect		
	n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	Change from Baseline Diff(95% CI)	n	Mean(SD)	Change from Baseline Diff(95% CI)
Baseline	41	0.69(0.34)		38	0.72(0.41)		79	-0.02(0.5)	
6 months	35	0.7(0.4)	-0.02(-0.15,0.1)	34	0.77(0.36)	0.05(-0.1,0.2)	69	0.05(0.53)	0.07(-0.13,0.26)
12 months	41	0.6(0.39)	-0.09(-0.2,0.03)	39	0.7(0.36)	-0.01(-0.16,0.13)	80	0.06(0.47)	0.08(-0.11,0.26)

Table 29. NT proBNP levels in placebo and TA-65 arms at baseline, 6 months and 12 months

4.13 Adverse events and clinical outcomes

There were 315 AEs reported, with fewer in the TA-65MD group compared to placebo (130 vs 185 AEs) **(Table 30)**. Most AEs were grade 1 (49.5%) or grade 2 (36.5%), were most commonly infectious, gastrointestinal or respiratory (**Table 31**), and most were unrelated to trial medication (89.8%) (**Table 32**). The number of serious AEs was similar in TA-65MD and placebo groups (19 vs 23). Fewer patients reported any grade 1-3 AEs in the TA-65MD group **(Table 33)**. One patient had a grade 4 AE (respiratory failure, which resolved) and one patient died of acute myeloid leukaemia – both in the TA-65MD group but were unrelated to treatment.

Adverse Event Severity	Placebo	TA-65	Total
All Adverse Events (Grade 1-5)	185 (58.7%)	130 (41.3%)	315
Grade 1	91 (58.3%)	65 (41.7%)	156 (49.5%)
Grade 2	71 (61.7%)	44 (38.3%)	115 (36.5%)
Grade 3	23 (54.8%)	19 (45.2%)	42 (13.3%)
Grade 4	0	1 (100%)	1 (0.3%)
Grade 5	0	1 (100%)	1 (0.3%)

Table 30: Comparison of adverse events severity between treatment groups. Percentages given in placebo and TA-65 columns are as proportion of all AEs of that grade. Percentage given in Total column are as percentage of all AEs. Definition of severity grade is given in the main text.

AE classified according to organ system	Total
Cardiac	35
Respiratory	41
Vascular	11
Gastrointestinal	38
Infection	46
Nervous	19
Musculoskeletal	38
Skin	20
Psychiatric	15
Blood and lymphatics	13
Renal	6

AE classified according to organ system	Total
Other	33
Total	**315**

Table 31. Total number of AEs classified according to organ system

Adverse Event Causality	Placebo (n=185)	TA-65 (n=130)	Total (n=315)
Possibly related	6	3	9
Unlikely to be related	17	6	23
Unrelated	162	121	283

Table 32. Summary of AE causality. Of 9 AEs possibly related to IMP, 8 were grade 1 severity and 1 was grade 3 severity (hospitalisation due to severe anxiety).

Adverse Event Severity	Placebo (n=45)	TA-65 (n=45)
Grade 1	36 (80%)	31(68.9%)
Grade 2	27 (60%)	24 (53.3%)
Grade 3	10 (22.2%)	7 (15.6%)
Grade 4	0 (0%)	1 (2.2%)
Grade 5	0 (0%)	1 (2.2%)

Table 33. Comparison of adverse event severity per patient between treatment groups. The number of patients in each treatment group reporting at least 1 AE of each grade.

4.14 Adherence

Adherence to trial medications were similar in both groups between month 1 and month 12 (**Table 34**).

Adherence 80% allocated doses	Placebo	TA-65
Month 1	42 (93.3%)	44 (97.8%)
Month 3	40 (88.9%)	43 (95.6%)
Month 6	40 (88.9%)	38 (84.4%)
Month 9	39 (86.7%)	38 (84.4%)
Month 12	39 (86.7%)	37 (82.2%)

Table 34. Adherence to at least 80% of allocated drug doses.

Chapter 5
Discussion

5.1 Impact on primary endpoint

The primary endpoint of the trial, the percentage of $CD8^+$ T_{EMRA} did not differ between the 2 arms. $CD8^+$ T_{EMRA} levels increased in the placebo group from 59% to 61% and TA-65 group from 58% to 61%. Examination of subgroup analysis by CMV and ACS type did not show any significant treatment effect of TA-65 on $CD8^+$ T_{EMRA} levels. There were no treatment effect and no significant changes from baseline to 12 months in both NSTEMI and STEMI groups.

In the absence of an established biomarker of immunosenescence, we selected % $CD8^+$ T_{EMRA} which epitomises immune dysregulation associated with increased clinical events such as ischemic stroke or MI. Indeed, Martin-Ruiz et al reported percentage of $CD8^+$ T_{EMRA} as an independent predictor of all-cause mortality in octogenarians after adjustment of known risk factors such as heart failure, cancer and frailty. The population of $CD8^+$ T_{EMRA} is prominent among the elderly population. TEMRA cells accumulate faster in the CD8 versus CD4 populations. In our study, $CD8^+$ T_{EMRA} was defined as CD8 T cells lacking C-C chemokine receptor type 7 (CCR7) and re-expressing CD45RA. CCR7 is a chemokine receptor that controls homing to secondary lymphoid organs. CCR7- cells have immediate effector function and express receptors promoting migration to sites of inflammation. In contrast, CCR7+ cells display lymph node homing receptors. T cells that re-express CD45RA are characterised by low proliferative activity, high levels of DNA damage and loss of telomerase activity[106]. $CD8^+$ T_{EMRA} accumulation is likely precipitated by chronic viral infection namely CMV. Our trial confirmed that CMV positive patients had significantly higher levels of $CD8^+$ T_{EMRA} cells compared to CMV negative patients. However, we did not assess SASP markers including chemokines, cytokines and proteases. Cells measured in peripheral blood may not reflect the populations present in the atherosclerotic lesions. In carotid artery plaques of symptomatic patients, there were more activated, differentiated and exhausted T cells compared to their blood counterparts[197].

Hoffmann et al have reported on loss of $CD8^+$ T_{EMRA} post acute MI. The decline was notable in the first 30 minutes post reperfusion especially in CMV positive patients. The cells replenished at 24 hours but remained lower compared to health controls and patients with MI 3 months ago. In our study, median time from reperfusion to blood sampling was 13 days and hence, we expect CD8 TEMRA levels to stabilise after the initial acute trough. T cell depletion primarily due to an acute decrease in both CD4 and CD8 effector cells (TEM and TEMRA) was further demonstrated in 59

prospectively recruited STEMI patients undergoing PPCI[5]. The effector cells migrate to the infarct zone releasing proinflammatory cytokines IFN-γ and TNF-a. The loss of effector T cells was notably seen only in STEMI and not in NSTEMI. In our study, there was a large time variation in infarct to baseline blood sampling, thus precluding analysis of potential differences between STEMI and NSTEMI.

5.2 Impact on lymphocyte subsets

Our study showed that patients treated with TA-65 had an increase in the number of all lymphocyte subsets. An increased lymphocyte count enhances adaptive immunity and provides resilience against infection, cancer and cardiovascular disease [198]. Conversely, experimental studies have showed that lymphopenia provokes a dysregulated expansion of memory T cells which may induce a proinflammatory milieu and increase the risk of cardiovascular disease. Subsequently, observational studies have confirmed an association between lymphopenia and adverse cardiac outcomes. In a large retrospective cohort of over 31000 healthy Americans, relative lymphopenia <1500/uL and severe lymphopenia <= 1000/uL were associated with increased all-cause mortality - age and sex adjusted HR 1.3 (95% 1.2-1.4) and 1.8 (95% CI 1.6-2.1) respectively[199] In over 15000 patients undergoing coronary angiography, relative lymphopenia and severe lymphopenia were associated with increased mortality[200]. Moreover, lymphopenia following PPCI confers poor prognosis. In a Cox regression analysis, low lymphocyte <1300 cells/uL (versus high >1910) was an independent predictor of mortality (HR 2.42, 95% CI 1.25-4.71)[5]. In the current pandemic, elderly patients with coronary artery disease are susceptible to adverse outcomes with COVID-19 infection [201]. Lymphopenia at presentation is strongly predictive of mortality and is driven by T cell depletion. In particular, T cell immunity is impaired in the elderly [202].

Of note, our study showed an increase in cytotoxic CD4CD28- and CD8CD28- T cells with TA-65 treatment. While their proinflammatory characteristics may pose a concern after ACS, these cells are important components in eradicating cells with malignant potential or infected with CMV. Considering evolving evidence, improving lymphocyte counts after ACS may improve clinical outcomes. With elderly cardiac patients especially vulnerable to COVID-19, improving immunity with TA-65 warrants further study.

5.3 Inflammation as a residual risk post ACS

In current clinical practice, secondary prevention post ACS is dominated by lipid-lowering and antiplatelet therapies. However, active inflammation is a key pathological process in both atherosclerosis and ACS, thus representing an unmet therapeutic need. The CANTOS trial identified inflammation, as quantified by hsCRP, as a key determinant of residual risk. In patients with coronary artery disease and elevated hsCRP (>2 mg/l), targeting the IL-1B pathway led to a reduction in the rate of major adverse cardiovascular events. In a prespecified secondary analysis, patients with the highest on-treatment reduction in hsCRP derived the most benefit in terms of reduction in cardiovascular event rate [203]. A reduction in hsCRP to below 1.8mg/L defined a lower risk group.

Baseline hsCRP was higher in the STEMI versus NSTEMI group. This is related to the larger inflammatory response in STEMI and greater infarct size. In the TACTIC trial, the mean hsCRP was 62% lower in the treatment group (1.1 mg/L v 2.9 mg/L) at 12 months, suggesting TA-65 reclassified patients into a lower risk group. The reduction in hsCRP with TA-65 was even more pronounced in STEMI patients, at 72%. The differences were not statistically significant since the trial was not powered for examining effect on hsCRP.

Reducing inflammation after MI is expected to improve outcomes through the following mechanisms. Firstly, MI has been shown to accelerate the progression of coronary atherosclerosis even in the non-culprit artery. Experimental studies have shown that focal arterial inflammation in response to vascular injury increased systemic vascular inflammation and accelerated remote atheroma progression [204]. After MI, APOE-/- mice developed more advanced atherosclerotic lesions associated with marked increase in monocyte recruitment. Following MI, haematopoietic stem cells and progenitor cells were released from the bone marrow and then seeded to the spleen, producing sustained monocyte production [205].

Secondly, adverse left ventricular remodelling is triggered by the intense acute inflammatory response following MI. Adverse remodelling is a critical cause of heart failure post MI and is associated with worse outcomes. Long term mortality rate in the era of primary angioplasty remains significant- approximately 7 %[206]. Experimental studies have shown that reducing inflammation, through recruitment of regulatory T cells, is associated with reduced adverse remodelling of the infarcted heart[207]. A pooled analysis of 3 early phase randomised clinical trials has demonstrated a reduction in heart failure with the IL-1 antagonist anakinra[208]. Anakinra does not appear to have an increased risk of severe infections.

5.4 Limitations of current anti-inflammatory therapy

Inflammation poses a residual risk post ACS that is not fully addressed by contemporary therapy. Moreover, PCI results in endothelial injury which may provoke an inflammatory additional response. Colchicine, a low cost anti-inflammatory drug, has been scrutinised as a potential addition to the ACS treatment armamentarium. A recent meta-analysis of 7 randomised controlled trials comparing colchicine and placebo in 6660 patients undergoing PCI showed significantly reduced MACE outcomes (RR 0.73 95% C 0.61-0.87) driven by the reduction in repeat vessel revascularisation, stroke and stent thrombosis[209]. However, a focused meta-analysis demonstrated a significant increase of non-CV death among patients treated with colchicine versus placebo at an average follow up of 25 months (OR 1.55, 95% CI 1.10-2.17). The main drawback of canakinumab used in the CANTOS trial was the adverse impact of IL-1 blockage on immunity. Patients treated with canakinumab were more likely to succumb to sepsis.

In the TACTIC trial, the fact that patients receiving TA-65 exhibited no significant difference in myeloid cells (monocytes and neutrophils) compared to placebo suggests that their innate immunity was not compromised. Indeed, a rise in the number of all lymphocyte subsets suggests that their adaptive immunity may be improved.

5.5 Effect on heart failure parameters

Post MI, approximately 25% of patients are at risk of a heart failure event at one year despite contemporary treatment. In the post MI population, angiotensin receptor-neprilysin inhibitor (ARNIs) compared to ACE-inhibitor has not been shown to reduce mortality or hospitalisation for heart failure [210]. Hence, there is an unmet need to reduce adverse events in patients following large infarcts. The TA-65 group had lower NT-proBNP after 12 months (299 vs 520pg/mL), but the treatment effect was not statistically significant, possibly due to low sample size.

Studies have shown an association between telomere dynamics and cardiomyocyte biology. Cardiomyocyte telomeres of patients with heart failure were shorter than those of healthy donor hearts independent of age[211]. Since there was no difference in the length of telomeres in the smooth muscle cells between failing and healthy hearts, this seemed to be a unique hallmark in cardiomyocytes. Telomere shortening, reduced cardiomyocyte proliferation, increased cardiomyocyte death and increased cardiac

remodelling were all observed in the telomerase knockout mice hearts with ageing[212]. On the other hand, increased expression of TERT led to increased proliferation, hypertrophy and survival of rat cardiomyocytes while maintaining telomere length[213]. In heart failure patients, the association between leukocyte telomere length and clinical outcomes is uncertain. In a large study including more than 3600 patients with different heart failure phenotypes, mean circulating leukocyte telomere length was independently associated with all-cause mortality but not heart failure hospitalisation[126]. The association of shorter LTL was stronger for non-CV than CV-related mortality. In the Framingham Heart Study, the strongest association to mortality was not attributed to CV causes. These findings suggest that the association with mortality may not be related to an impact of shorter telomeres on cardiac function. In our study, we did not measure telomere length in circulating leucocytes. While this fails to investigate the canonical effect of telomerase activation, there is a lack of causal relationship between reduced leucocyte TL and coronary artery disease or heart failure. Moreover, leucocyte telomere length may not reflect TL in cardiac-specific cells.

5.6 Effect on endothelial function

Our study did not demonstrate any significant effect of TA-65 on peripheral endothelial function. In TACTIC, over 90% of patients had RHI greater than 1.35 which is interpreted as unlikely to have significant coronary endothelial dysfunction. This is a surprising finding in patients with established CAD. Endothelial dysfunction is a precursor to clinical symptoms of CVD and serves as an index encompassing the entire stress imposed by cardiovascular risk factors [214]. Hence, in largely untreated population at risk of CV disease, endothelial dysfunction is associated with adverse outcomes [188]. However, the utility of evaluating endothelial vasodilatory function in patients with advanced coronary artery disease, such as those in TACTIC, is unclear. Moreover, most patients were established on ACE-inhibitors which can rapidly reverse impairment of endothelium-dependent vasodilation in atherosclerosis[215]. Hence, in patients with contemporary secondary prevention, the additional impact of telomerase activation on endothelial function may be negligible. Stopping ACE-inhibitors in high-risk post ACS patients to allow a wash out period prior to endothelial function measurement would not have been clinically appropriate.

RH-PAT is an emerging tool for serial assessment of endothelial function to investigate the impact of pharmacological intervention and does not yet have the evidence base of the more traditional brachial FMD. However, it is considered less technically challenging and operator dependent. Studies assessing digital RHI and brachial FMD simultaneously found conflicting results. In healthy individuals and patients with CAD, there was no association between RH-PAT ratio brachial artery flow-mediated dilation[216]. In a small study of patients with CAD, most patients had abnormal FMD but normal RHI [217]. In a large, community-based sample, brachial and digital measures of vascular function had conflicting associations with cardiovascular risk factors [218]. Notably, abnormal FMD but not PAT ratio was more prevalent with increasing age. FMD and RH-PAT assess endothelial function of distinct vascular phenotype. While TA-65 did not affect microcirculatory endothelial function, its impact on the conduit arteries remains uncertain. Moreover, the utility of RH-PAT as a surrogate of coronary endothelial function in elderly patients with confirmed CAD warrants further study.

5.7 Mechanistic insights

Telomerase activity in PBMCs was measured using innovate droplet digital PCR. No difference was detected between groups. TA-65 may activate telomerase in more central tissues such as bone marrow, thymus and spleen, rather than in peripheral blood cells. Moreover, it is most likely to affect proliferating lymphocytes which are a small proportion of total lymphocytes.

Hence, it is unclear exactly how TA-65 elicits an increase in lymphocytes, while also reducing inflammation. The proliferation of cultured splenocytes is considerably increased by TA-65 in a dose-dependent manner and TERT is a key regulator of lymphocyte proliferation [219]. This enhanced proliferation was likely caused by TERT since splenocytes lacking TERT expression were unaffected by TA-65 stimulation. However this study assessed higher doses in vitro than would be expected in vivo with TA-65. The finding that TA-65 does not affect lymphocyte telomerase activity suggests that TA-65 does not increase lymphocyte numbers via classical telomere maintenance and nuclear telomerase activation. Our findings are consistent with those of Ale-Agha et al, who showed that TA-65 exclusively elevates mitochondrial rather than nuclear TERT expression [220]. However, since mitochondrial failure can be a direct cause of cellular senescence [221], TA-65 still

has the potential to have an impact. It has been demonstrated that ageing causes a reduction in mitochondrial activity of many cell types, including T-lymphocytes[222]. Despite the lack of direct evidence, global TERT activation inhibits senescence [223] and mitochondrial TERT binds to and protects mitochondrial DNA, which may help to maintain the function of the electron transport chain while reducing mitochondrial ROS, a major senescence inducer [224]. Senescence accumulation can cause phenotypic alterations linked to CVD, including atherosclerosis and hypertension [225], [226]. Reducing senescence is linked to the delaying, preventing or reversing of characteristics of CVD, implying that senescence is not just a response to disease but rather contributes to both their genesis and progression [227]–[230]. Hence, regardless of telomere maintenance, prevention of senescence via enhanced mitochondrial activity may contribute to a reduction in cardiac events in the treated cohort.

Senescent cells contribute to disease pathogenesis by expressing the SASP, a pro-inflammatory cocktail that directly connects mitochondrial metabolism to pro-inflammatory cellular responses [231]. Therefore, diminished SASP generation and enhanced mitochondrial activity may aid in mitigating SASP production and partially account for our observations of decreased inflammation in the TA-65 treated sample. Another possibility is that TA-65 stimulates regulatory CD4+ T lymphocytes (Tregs) which prevent macrophage activation and release of IL-1, IL-6, and hsCRP [232]. To identify a potential mechanistic connection between the rise in lymphocyte populations and decrease in inflammation, additional high-resolution immunophenotyping research, such as that using spectral cytometry, is required [233].

5.8 Strengths and Limitations

TACTIC was a pilot study designed to provide preliminary evidence on the efficacy of TA-65 in reversing immunosenescence in elderly patients after ACS. The double-blinded randomised placebo-controlled trial design provided quality assurance for evaluation of an IMP. The trial included older patients who are underrepresented in many major ACS clinical trials[234]. The following factors are considered barriers to their participation in research (1) cognitive impairment which precludes informed consent (2) a high prevalence of baseline comorbidity and polypharmacy, and (3) multifactorial loss to follow-up[234]. This potentially compromises the applicability of evidence-based therapies to this high risk group. In TACTIC, 60% of patients screened

declined to participate. The trial demanded a high level of commitment with multiple in-person visits over one year. Due to the large geography served by JCUH, many patients also needed to travel long distances for their research appointments.

Eleven patients stopped IMP mainly due to personal choice or adverse events all unlikely related to IMP. Three patients were diagnosed with cancer (bowel, metastatic lung and acute myeloid leukaemia) during the trial and therefore stopped IMP, and one further patient had a previously undeclared low grade prostate cancer (under surveillance). These rates of cancer are within expected rates in the 12 months following MI in this population, estimated at 3 per 100 patient years.[235] The rate of new diagnosis of cancer is thought to be related to increased surveillance, prothrombotic tendency in cancer patients, a high prevalence of risk factors, advanced age, and the unmasking of previously asymptomatic disease (such as bleeding from bowel or lung cancer in patients in the TACTIC trial). TACTIC had lower rates of permanent drug discontinuation (17%) compared to most major cardiovascular trials from the TIMI group[236]. There was >85 % adherence to least 80% of allocated drug doses. There was no difference in treatment effect on CD8+ T proportion when accounting for non-compliers using the CACE analysis.

We selected CD8 TEMRA proportion as the primary endpoint due to its highly senescent properties and adverse prognostic implications. However, emerging evidence highlights the mitochondrial action of TA-65 and thus it likely targets multiple lymphocyte subsets as opposed to very specific ones. We measured immune cell counts in peripheral blood which may differ in coronary plaques [237]. Our trial did not have a healthy control. A direct comparison of T cell phenotype and function between ACS patients and matched healthy people would clarify whether TA-65 preserves lymphocyte count for the age group or accelerates its return to normal after MI.

5.9 Future research

We plan to measure leucocyte telomere length on stored samples to assess the canonical effect of telomerase activation. Compared to baseline leucocyte TL, rate of telomere shortening has a stronger association with outcomes[238]. We demonstrated a reduction in hsCRP which is a downstream marker of systemic inflammation. Measuring systemic cytokine levels such as IFN-γ would provide in-depth understanding of the impact of telomerase activation on SASP. We hypothesize that improved mitochondrial function in T lymphocytes leads to a reduction in cytokine generation and hence reduced inflammation

Finally, the effects of TA-65 on lymphocyte count elevation and hsCRP reduction warrant an adequately powered study. Moreover, the full potential of TA-65 may not be achieved at the dose tested (8 mg BD) and a future study will have a separate higher dose arm of 16 mg BD.

Conclusions

TA-65 was associated with an increase in total lymphocytes, CD4 and CD8 T lymphocytes at 12 months while no difference was observed in monocytes and neutrophils. TA-65 was associated with a trend for lower hsCRP on patients already on high dose statins. While a causal relationship cannot be ascertained from this pilot trial, a mechanism is tentatively debated. Nuclear telomerase activation appears unlikely since naïve and differentiated cells did not differ in their response to TA-65. We speculate that TA-65 is a mitochondrial telomerase activator. Improving mitochondrial biogenesis and metabolism may improve cell survival and reduce SASP expression. This in turn reduces inflammation. It is well documented that acute and chronic inflammation lead to retention of lymphocytes in either lymphatic tissues, adherence to endothelium in venous circulation, or transmigration to inflamed tissues. Reduced inflammation may therefore contribute to a higher number of lymphocytes in the circulation. The finding of reduced inflammation and increased adaptive cell immunity is promising and may address a treatment gap in patients post MI. The effect of TA-65 on hsCRP reduction, a clinically relevant therapeutic target post MI, should be confirmed in an adequately powered trial. Finally, future studies using mitochondrial functional assays in stored PBMCs are warranted to clarify the mechanism of TA-65.

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Appendix

Supplemental table 1. Comparison of baseline characteristics between CMV negative and CMV positive patients.

	CMV negative (N=24)	CMV positive (N=66)	P value
Age, years (mean)	71.4	71.2	0.899
Male (%)	95.8	80.3	0.072
Family History (%)	45.8	42.4	0.773
Current smoker (%)	12.5	9.1	0.867
Ex smoker (%)	58.3	63.6	0.867
Hypertension (%)	58.3	47.0	0.340
Hyperlipidaemia (%)	66.7	63.6	0.791
Diabetes mellitus (%)	25.0	10.6	0.086
Previous MI (%)	16.7	18.2	0.868
Previous PCI (%)	12.5	15.2	0.752
Previous CABG (%)	20.8	4.5	0.016
PVD (%)	8.3	6.1	0.702
CVA (%)	8.3	7.6	0.906
Total chol, mmol/L (mean)	4.1	4.6	0.166
Creatinine, mmol/L (mean)	95	83	0.019
Peak troponin, ng/L (mean)	9554	10907	0.610
STEMI (%)	45.8	48.5	0.824
NSTEMI (%)	54.2	51.5	0.824
MVD (2VD or more) (%)	39.1	46.2	0.560
Baseline results			
EndoPAT	1.74	1.94	0.125
CD8_TEMRA (%)	46.2	68.3	<0.001
CD8_CD28- (%)	40.9	65.6	<0.001
CD4_CD28- (%)	2.5	9.9	0.006
CD4:CD8 ratio	3.9	2.5	0.019