

BMJ Open

BMJ Open is committed to open peer review. As part of this commitment we make the peer review history of every article we publish publicly available.

When an article is published we post the peer reviewers' comments and the authors' responses online. We also post the versions of the paper that were used during peer review. These are the versions that the peer review comments apply to.

The versions of the paper that follow are the versions that were submitted during the peer review process. They are not the versions of record or the final published versions. They should not be cited or distributed as the published version of this manuscript.

BMJ Open is an open access journal and the full, final, typeset and author-corrected version of record of the manuscript is available on our site with no access controls, subscription charges or pay-per-view fees (<http://bmjopen.bmj.com>).

If you have any questions on BMJ Open's open peer review process please email info.bmjopen@bmj.com

BMJ Open

Biobanking for discovery of novel cardiovascular biomarkers using imaging-quantified disease burden: protocol for the longitudinal, prospective, BioHEART-CT cohort study

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2018-028649
Article Type:	Protocol
Date Submitted by the Author:	18-Dec-2018
Complete List of Authors:	<p>Kott, Katharine; University of Sydney, Sydney Medical School, Faculty of Medicine and Health Vernon, Stephen; University of Sydney, Sydney Medical School, Faculty of Medicine and Health Hansen, Thomas ; Kolling Institute of Medical Research Yu, Christine; Kolling Institute of Medical Research Bubb, Kristen ; University of Sydney, Northern Clinical School, Faculty of Medicine and Health Coffey, Sean; Royal North Shore Hospital, Kolling Institute and Department of Cardiology; University of Otago, Dunedin School of Medicine Sullivan, David; The University of Sydney Yang, Jean ; University of Sydney, School of Mathematics and Statistics O'Sullivan, John; University of Sydney, Sydney Medical School, Faculty of Medicine and Health Chow, Clara; The George Institute for Global Health and Westmead Hospital, Sydney Patel, Sanjay; University of Sydney, Sydney Medical School, Faculty of Medicine and Health Chong, James ; University of Sydney, Sydney Medical School, Faculty of Medicine and Health Celermajer, David; University of Sydney, Faculty of Medicine and Health; Royal Prince Alfred Hospital, Department of Cardiology Kritharides, Leonard; Concord Hospital, Cardiology Grieve, Stuart; University of Sydney, Sydney Medical School, Faculty of Medicine and Health Figtree, Gemma; University of Sydney, Faculty of Medicine and Health; Kolling Institute of Medical Research,</p>
Keywords:	Coronary heart disease < CARDIOLOGY, Cardiovascular imaging < RADIOLOGY & IMAGING, Ischaemic heart disease < CARDIOLOGY, Risk management < HEALTH SERVICES ADMINISTRATION & MANAGEMENT, MOLECULAR BIOLOGY, Clinical chemistry < PATHOLOGY



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

BMJ Open: first published as 10.1136/bmjopen-2018-028649 on 18 September 2019. Downloaded from <http://bmjopen.bmj.com/> on April 23, 2024 by guest. Protected by copyright.

Biobanking for discovery of novel cardiovascular biomarkers using imaging-quantified disease burden: protocol for the longitudinal, prospective, BioHEART-CT cohort study

Katharine A. Kott^{*1,2}, Stephen T. Vernon^{*1,2}, Thomas Hansen¹, Christine Yu¹, Kristen J. Bubb¹, Sean Coffey^{1,5}, David Sullivan^{2,3,6}, Jean Y. Yang^{3,4}, John F. O'Sullivan^{2,3,9}, Clara K Chow^{2,10,13}, Sanjay Patel^{2,3,7,9}, James Chong^{2,10,11,12}, David S. Celermajer^{2,7,9}, Leonard Kritharides^{2,11}, Stuart M. Grieve^{2,3,8}, Gemma A. Figtree^{1,2}

¹Cardiothoracic and Vascular Health, Kolling Institute and Department of Cardiology, Royal North Shore Hospital, Northern Sydney Local Health District, Australia

²Sydney Medical School, Faculty of Medicine and Health, University of Sydney, Australia

³Charles Perkins Centre, University of Sydney, Australia

⁴School of Mathematics and Statistics, University of Sydney, Australia

⁵Dunedin School of Medicine, University of Otago, New Zealand

⁶Department of Biochemistry, Royal Prince Alfred Hospital, Sydney, Australia

⁷Department of Cardiology, Royal Prince Alfred Hospital, Sydney, Australia

⁸Department of Radiology, Royal Prince Alfred Hospital, Sydney, Australia

⁹The Heart Research Institute, Sydney, Australia

¹⁰Department of Cardiology, Westmead Hospital, Sydney, Australia

¹¹ANZAC Research Institute & Cardiology Department, Concord Repatriation General Hospital, Sydney, Australia

¹²Westmead Institute for Medical Research, Westmead Hospital, Sydney, Australia

¹³Westmead Applied Research Centre, University of Sydney, Sydney, Australia

Corresponding author:

Professor Gemma Figtree MBBS DPhil (Oxon) FRACP FAHA

Cardiothoracic and Vascular Health, Level 12, Kolling Institute of Medical Research, Royal North Shore Hospital, St Leonards, NSW, 2065, Australia. Email: gemma.figtree@sydney.edu.au.

Abstract

Introduction: Coronary artery disease (CAD) persists as a major cause of morbidity and mortality worldwide despite intensive identification and treatment of traditional risk factors. Data emerging over the past decade shows a quarter of patients have disease in the absence of any known risk factor, and half have only one traditional risk factor. Improvements in quantification and characterisation of coronary atherosclerosis by CT coronary angiography (CTCA) can provide quantitative measures of subclinical atherosclerosis - enhancing the power of unbiased “omics” studies to unravel the missing biology of personal susceptibility, identify new biomarkers for early diagnosis, and to suggest new targeted therapeutics.

Methods & Analysis: BioHEART-CT is a longitudinal, prospective cohort study, aiming to recruit 5000 adult patients undergoing clinically-indicated CTCA. After informed consent, patient data, blood samples and CTCA imaging data are recorded. Follow-up for all patients is conducted one month after recruitment, and then annually for the life of the study. CTCA data provides volumetric quantification of total calcified and non-calcified plaque, and measures that characterise plaque vulnerability. Comprehensive molecular phenotyping will be performed using state-of-the-art genomics, metabolomics, proteomics, and immunophenotyping. Complex network and machine learning approaches will be applied to biological and clinical datasets to identify novel pathophysiological pathways and to prioritise new biomarkers. Discovery analysis will be performed in the first 1000 patients of BioHEART-CT, with validation analysis in the following 4000 patients. Outcome data will be utilised to build improved risk models for CAD.

Ethics & Dissemination: The study protocol has been approved by the human research ethics committee of North Shore Local Health District in Sydney, Australia. All findings will be published in peer-reviewed journals or at scientific conferences.

Registration: This study is registered with the Australia and New Zealand Clinical Trials Registry - ACTRN12618001322224.

Article Summary

Strengths and Limitations of this Study (max five bullet points)

- BioHEART-CT is a prospective, longitudinal cohort study assessing patients with suspected CAD undergoing CTCA across multiple Australian centres.
- Quantitative measures of coronary atherosclerosis from CTCA datasets will be used in conjunction with biological samples, improving the power to discover new mechanisms and markers for CAD.
- Samples stratified by imaging-quantified disease severity will be analysed by both candidate and unbiased “omics” approaches, utilising modern technologies and bioinformatic analysis to discover new biomarkers using proteomics, metabolomics, lipidomics, transcriptomics, genomics and immunophenotyping.
- Longitudinal outcome data will be used to build risk models which can incorporate both traditional and novel risk factors and biomarkers.
- Limitations of the study include selection bias, as only patients with a clinical indication for CTCA are included, and the geographic isolation of Australia, which may result in the need for confirmation of results in multi-national studies.

Introduction

Cardiovascular disease has persisted as a major cause of human morbidity and mortality despite continual improvements in preventative therapies. In 2015 cardiovascular disease accounted for one-third of all deaths world-wide¹, and ischaemic heart disease (IHD) remains the leading cause of years of life lost for high and middle sociodemographic groups multi-nationally². This large burden of disease puts significant stress on the health systems of individual countries. In Australia, cardiovascular disease remains the number one killer of Australians³, with local health costs estimated to be \$7.7 billion AUD per annum⁴.

The standard modifiable cardiovascular risk factors (SMuRFs⁵) for atherosclerosis - hypertension, diabetes mellitus, hyperlipidaemia and smoking - were identified in epidemiological studies in the 1960s⁶. The identification and subsequent efforts to target these risk factors at community and primary care levels have led to a substantial reduction in mortality from cardiovascular disease¹. However, substantial disease burden remains and importantly, individual variability in susceptibility to these risk factors is considerable. While continuing our efforts to tackle societal and modifiable risk factors, identifying undiscovered mechanisms that lead to the development of atherosclerosis is critical. Such work will provide new biomarkers for early detection of subclinical atherosclerosis and open avenues for improved preventative and therapeutic strategies.

The importance of new detection mechanisms for subclinical atherosclerosis is highlighted by interrogating the “fine print” of clinical studies, including information not usually presented in the main tables of manuscripts. Although data regarding the percentage of patients suffering myocardial infarction despite having no SMuRFs is often omitted, a large meta-analysis found that >50% of women and >60% of men presenting with their first ACS had 0 or 1 SMuRF⁷, and similar proportions of SMuRFless patients have been identified in ACS cohort studies^{5,8}. In a recent single centre Australian study, we showed that the proportion of patients presenting with ST-elevation myocardial infarction with 0 SMuRFs has risen from 11% to 27% over an 8 year period⁵. These patients have developed extensive CAD without having any of the red flags which would allow doctors to identify their risk of disease and intervene early. Although this is a relatively small piece of the overall IHD pie, the global burden of CAD is immense with an estimated prevalence of 470 million⁹, which makes this 25-30% SMuRFless patient population important from a public health perspective. It is sobering to think that we have made no clear advances that allow us to identify risk and subclinical disease in this subgroup. Indeed, we also have limited information regarding how these patients respond to traditional secondary preventative strategies, and whether their long term outcomes and disease progression differs from those with traditional modifiable risk factors.

Further evidence that novel biological mechanisms contribute substantially to atherosclerosis can be seen in results from large scale genome-wide association studies which have reported that 66% of the identified genes conveying heritable cardiovascular risk are not associated with the traditional risk factors¹⁰. Many of these genes were related to inflammatory processes, and studies such as the CANTOS trial¹¹ confirm the importance of inflammation in CAD. However, some of these important genes have not yet been well characterised, and are not yet associated with any known pathway in atherosclerosis and CAD, highlighting the importance of ongoing discovery work.

To address this gap in our understanding of CAD pathophysiology, we have designed a unique cohort study with biobanked samples to facilitate investigation of novel cardiac risk factors and biomarkers. This biobank includes advanced, quantitative imaging measurements of coronary artery atherosclerosis to allow for accurate phenotyping of the patient groups. This overcomes a

1
2
3 weaknesses of previous genomics studies that have relied on the presence or absence of a coronary
4 artery event to classify individuals as diseased or as a control, improving the statistical power of the
5 subsequent analysis. The advanced imaging dataset and clinical data including baseline and follow-
6 up are integrated with the collection of a broad range of biological samples for discovery work,
7 utilising state-of-the-art molecular phenotyping platforms and computational bioinformatics.
8
9

10 Objectives

11 The primary objectives of the BioHEART-CT study are:

- 12 1. To identify new biomarkers to assist in early CAD risk identification and stratification;
- 13 2. To identify new mechanistic pathways that may be targeted with novel therapeutic
14 strategies to abrogate CAD risk.
15
16

17 The secondary objectives of the BioHEART-CT study include the following:

- 18 1. To determine the predictive value of a modified CTCA scoring system for CAD risk which
19 incorporates plaque composition data;
- 20 2. To identify new risk factors for CAD based on integration of clinical information profiles,
21 CTCA results and clinical outcomes;
- 22 3. To develop a novel risk scoring system incorporating clinical risk factors, novel
23 biomarkers and CTCA scores to more accurately predict CVD events.
24
25
26
27
28

29 Methods and Analysis

30 Study Type

31 BioHEART-CT is a multicentre, prospective cohort study of patients with suspected CAD, which brings
32 together detailed clinical information with advanced imaging and molecular phenotyping.
33
34

35 Study Population

36 5000 patients will be recruited from participating hospitals' and associated imaging sites. All
37 recruitment centres are large tertiary hospitals within the city of Sydney, Australia.
38
39

40 Inclusion criteria:

- 41 - Patients who have been referred for investigation of suspected CAD
- 42 - Age 18 years or older
- 43 - Willing and able to provide informed consent
44
45
46

47 Exclusion criteria:

- 48 - Patients highly dependent on medical care who are unable to provide informed consent
- 49 - Patients unwilling or unable to participate in ongoing follow-up
50
51

52 Study Protocol

53 Screening and Enrolment

54 Eligible patients having a CTCA are invited to participate in the study. Eligibility is confirmed
55 according to the inclusion and exclusion criteria, the study is explained, and informed consent
56 obtained.
57
58
59
60

Patient Data Collection

The baseline questionnaire is completed by research staff conducting a face-to-face patient interview. Data obtained includes the following information: demographic data including smoking and alcohol intake, past medical history, relevant family history, medication history, history of cardiac symptoms and indication for CTCA. Data is entered into secure, encrypted databases in a de-identified format. After recruitment all patients are assigned a unique study number and all samples, imaging data and demographic information is identified by this number. The master list including identifiable data is securely stored on an encrypted server and is available only to senior research staff. Relevant details are made available to research staff for follow-up purposes.

Follow-Up & Outcome Adjudication

Participants are contacted by phone at approximately 30 days and then annually to determine if any events have occurred. Information recorded for any potential outcome event includes the acuity, need for hospitalisation and indication for admission, hospital attended, treating physician, date of admission and details of the events. New medical or surgical diagnoses since last contact are also recorded. Participants in BioHEART also consent to review of their electronic medical records and data linkage through the Centre for Health Record Linkage (CHeReL), which will be used for independent medical record reviews.

Significant outcome events that trigger a file review to assess for major adverse cardiovascular events (MACE) or secondary outcomes are the following:

- Unstable angina requiring hospitalisation
- Heart failure requiring hospitalisation
- Acute myocardial infarction
- Coronary angiogram with or without percutaneous intervention
- Cardiac bypass graft surgery
- Aortic or mitral valve surgery
- Cerebrovascular accident / transient ischaemic attack
- All-cause mortality

All medical records will be reviewed by an independent adjudication committee to determine if an event should be included in the study results. Detailed criteria for the individual diagnoses included in the outcomes must be met.

MACE is defined as cardiovascular death, non-fatal myocardial infarction or non-fatal stroke. Exploratory outcomes include revascularisation, unstable angina or heart failure requiring hospitalisation.

CTCA Data Acquisition

CTCA scans are obtained on 256 slice scanner, with reconstructions created using appropriate software for the individual machine. Each study is to be protocolled by a radiologist with at least level two accreditation (RANZCR/RANZCP). All radiographers are trained in acquisition and workup of CTCA. Oral metoprolol, or ivabradine if beta blockers are contraindicated, are given to optimise the heart rate if required. Dosing is adjusted by clinical staff according to baseline heart rate and bodyweight. Oral nitroglycerine (600-800 micrograms) is given immediately prior to the scan, and iodinated contrast is injected intravenously. Prospective studies are performed if the heart rate is

1
2
3 sufficiently controlled, otherwise retrospective acquisition is used. Average dose is minimised at all
4 participating sites in line with current recommendations^{12,13}.
5

6 CTCA Imaging Analysis

7
8 CT data is exported as thin DICOM data and is stored securely in a de-identified manner. Data is later
9 analysed using a dedicated workstation to obtain the primary clinical scores at each site. CTCAs are
10 analysed by standard anatomical arterial region as per the 17-segment model outlined by the
11 Society of Cardiovascular Computed Tomography¹⁴. Each segment is scored according to degree of
12 stenosis and composition of plaque (calcified, soft and mixed), see Figure 2. The segmental scores
13 are aggregated into a modified GENSINI score which represents the total amount of calcified and
14 non-calcified plaque present. Calcium scores are generated using the scanner-associated software by
15 the Agaston method. Total raw calcium scores, raw calcium scores for each vessel, and age and sex-
16 matched calcium percentage scores are recorded.
17
18

19
20 Further analysis will be performed at a central core laboratory using a Frontier System (syngo.via,
21 Siemens Healthcare, Erlangen), employing a semi-automated soft plaque algorithm.
22

23 Biological Sample Collection

24
25 A 20-30mL blood sample is taken from an in-situ intravenous cannula, or if this is not available, by
26 venesection, prior to CTCA and drug administration. Blood is collected into EDTA, serum and citrate
27 tubes and stored at 4°C until processing. A lithium heparin tube is collected and processed
28 immediately to extract peripheral blood mononuclear cells (PBMCs) from the buffy coat.
29

30 Sample Processing & Storage

31
32 Blood samples are processed by hospital pathology scientists experienced in laboratory procedures.
33 Briefly, whole blood is set aside, then tubes are centrifuged at 1861 x *g* for 15 minutes at 4°C prior to
34 aliquoting 500uL samples of serum, EDTA plasma, whole blood, buffy coat and erythrocytes. The
35 citrate tube is centrifuged at 1861 x *g* for a second 15 minutes at 4°C, after which citrate-plasma
36 samples are aliquoted. All aliquoted samples are frozen and stored at -80°C until analysis.
37
38

39
40 For PBMC isolation a standard gradient-separation protocol is used¹⁵. Briefly, blood from the lithium
41 heparin tube is diluted 1:1 with Hanks' Balanced Salt Solution (HBSS). Diluted whole blood is then
42 layered on top of Ficoll-Paque Plus and centrifuged (22°C, 1460 x *g* no brake, 20 min) . The buffy coat
43 layer containing PBMCs is then further purified with two wash steps in HBSS using repeat
44 centrifugation as above. PBMCs are then plated at a density of ~2.5 x 10⁴ cells/cm² into 0.1% gelatin-
45 coated flasks containing endothelial cell growth medium with 2% fetal bovine serum (EGM2
46 bulletkit, Lonza, Australia) and cultured in standard conditions for up to 21 days with regular
47 monitoring for the spontaneous growth of Endothelial Progenitor Cells (EPCs). Excess PBMCs are
48 immediately frozen in bovine serum (heat-inactivated, Gibco, Australia) containing 10% dimethyl
49 sulfoxide and stored in liquid nitrogen.
50
51

52 Sample Analysis

53
54 Samples will be analysed by a variety of methods with the intent of identifying candidate biomarkers
55 and novel metabolites involved in atherosclerosis pathophysiology. There will be two principal
56 approaches used to identify these factors.
57

58
59 First is a candidate approach, investigating currently identified factors that are thought to be
60 involved in human biological pathways that could predispose to atherosclerosis. These factors will be

1
2
3 assessed within the cohort by specific assays that relate to the biological pathways involved. The
4 candidate areas that have been identified for initial assessment are: soluble factors released by
5 inflammatory signalling within the atherosclerotic plaque, dysregulated redox signalling within the
6 endothelium, disorders of coagulation which lead to hypercoagulable states and/or hypofibrinolysis,
7 soluble platelet factors which indicate platelet dysfunction, and dysregulated signalling within
8 endothelial cells as evidenced by assessment of cultured EPCs derived from individual patient
9 samples. Individual assays have been designed to assess each candidate marker or pathway.
10

11
12 The second approach aims to investigate atherosclerosis biology in an unbiased manner by using
13 “omics” techniques. These assessments will utilise metabolomics, proteomics, transcriptomics,
14 genomics including SNP arrays and immunophenotyping techniques.
15

16 Proteomics for the cohort will be assessed utilising a clinical proteomics platform¹⁶ which will give a
17 complete description of the human peptidome in relevant samples, allowing for identification of
18 novel or common factors worthy of further investigation. Metabolomics and lipidomics will be
19 performed utilising six liquid chromatography-mass spectrometry platforms which will allow for
20 identification and quantitation of a broad spectrum of metabolites, including amino acids,
21 nucleotides, neurotransmitters and lipid subtypes¹⁷⁻²¹.
22

23
24 Immunophenotyping will be performed on PBMC samples via mass spectrometry time of flight
25 (CyTOF) which allows for detailed leucocyte phenotyping on an individual basis²². These profiles can
26 be associated with serum levels of established markers of inflammation such as VCAM-1, ICAM-1
27 and IL-6, as well as correlated with the balance of M1 and M2 phenotype monocytes which have a
28 well established role in atherosclerosis²³.
29

30
31 Transcriptomic and genomic assays will be utilised on subsets of the cohort of particular interest,
32 who for example may display particular resistance or vulnerability to CAD based on traditional risk
33 factor profile and disease burden. Whole transcriptome and RNA sequencing will be performed with
34 ribosomal depletion, and sequencing of the resulting reads and counting will be based on GENCODE.
35 Whole genome profiling will be performed on individuals with extreme athero-susceptible, or
36 athero-resilient phenotype, allowing for unbiased discovery of genetic variants that may be causally
37 associated with the extreme phenotypes²⁴.
38
39

40 Data Analysis Plan

41
42 Statistical machine learning approaches will be applied to these biological and clinical datasets, to
43 identify novel pathophysiological pathways, and to prioritise new markers based on likely causal
44 roles. Discovery analysis will be performed in the first 1000 patients of BioHEART-CT, with validation
45 in the following 4000 patients recruited, and in international collaborators studies (Figure 3.).
46 Sample size planning for developing classifiers using high dimensional data is based on NIH Biometric
47 online calculator assuming 1.3 fold standardised change, 50,000 molecular variables, and balanced
48 extreme groups.
49

50
51 Biomarker and risk factor data analysis will depend on the specifics of the assay or investigation
52 performed. Generally, continuous variables will be presented as means (with standard deviations) or
53 medians (with inter-quartile ranges), categorical variables as proportions (%) with chi-squared or
54 Fisher's exact test used to determine differences between groups. Hazard ratios for a one standard
55 deviation increase in a biomarker, after log transformation to remove effects of outliers, on MACE
56 will be obtained from weighted Cox regression models. All p-values reported will be two-sided, with
57 the 5% threshold used to determine significance.
58
59
60

1
2
3 For testing the prognostic value of prioritised biomarkers, and assuming a C-statistic of 0.75 for
4 traditional risk factors, we estimate that we will require 705 participants to detect a C-statistic of
5 0.85 with $p=0.05$, power of 80% and an event rate of 20%. Models developed with different sets of
6 variables and biomarkers and C-statistics for one year risk will be determined and compared. The
7 model's ability to discriminate and reclassify one year risk will be assessed using the integrated
8 discrimination index and the net reclassification improvement.
9
10

11 Discussion

12
13
14 Three main advances have been applied in the design of BioHEART-CT that provide strategic
15 advantage compared to previous studies which also used unbiased approaches to discover novel
16 mechanisms and markers of atherosclerosis. First, this study includes quantification and
17 characterisation of plaque (and the absence of plaque) by CT coronary angiography and advanced
18 imaging algorithms, improving the power of the study compared to those that relied on CAD-related
19 clinical events. Second, this study integrates high through-put, state-of-the-art multi-omic molecular
20 phenotyping with comprehensive clinical data and follow up. And third, the study brings together a
21 global team of biologists and bioinformaticians with complementary approaches and perspectives to
22 enhance the likelihood of new discoveries.
23
24

25
26 In previous CAD studies, healthy controls were often defined as patients who had not had a cardiac
27 event, or who had non-obstructive atherosclerosis on invasive coronary angiography which has
28 limited utility in identifying extraluminal plaque. As CAD often remains silent until it is very
29 advanced, those without a myocardial infarction could have quite extensive disease and still be
30 considered a "healthy" control. In BioHEART-CT, we have the capability to use our CTCA subgroup to
31 more precisely phenotype subjects, allowing the accurate creation of a group of healthy controls
32 which truly have no atherosclerotic disease present in their coronary arteries. This group of healthy
33 patients can be used as a comparator for those with various stages of disease, reducing confounding
34 factors and enhancing the capacity to detect real differences in biology.
35
36

37
38 Additionally, CTCA phenotyping can be utilised to analyse plaque composition in detail. Using scoring
39 systems such as GENSINI²⁵, severity scores can be generated for each individual. We have also
40 developed a modified GENSINI score as outlined above, which gives different weight to the presence
41 of older, calcified plaque and newer, more biologically active non-calcified plaque. Automated
42 detection of soft plaque shows promise as a scalable, reproducible method to add value to existing
43 CTCA analysis²⁶. In identifying patients with high levels of active atherosclerotic plaque, we can
44 create groups enriched for this biological feature, with and without traditional risk factors. This will
45 better enable us to detect novel mechanisms of CAD in those with unexplained susceptibility.
46
47

48
49 Our unbiased approach to cardiovascular risk factor discovery, applying the advantages of high
50 throughput multi-omics platforms, improved quantification of plaque and its characteristics, and
51 advanced statistical bioinformatics, provides a high chance of discovering previously unidentified
52 mechanisms and markers of disease. We have recently discussed the advantages of integrating
53 multi-omics over isolated genomics studies for the complex disease process of atherosclerosis²⁷, and
54 this will be particularly beneficial in such well-phenotyped groups.
55

56
57 Molecular markers found to be associated with atherosclerosis may be direct drivers of disease, or
58 result from the plaque and dysregulated vascular physiology. The closer the marker is to the
59 underlying biology, the more likely it will be to be useful in clinical practice, transforming our ability
60 to identify early vascular disease and susceptibility above and beyond traditional risk calculators. We
are well placed to take any markers and their surrounding pathways into preclinical cellular and

1
2
3 animal models to determine causality and/or mechanisms for association. This will help prioritise
4 markers for testing in prospective clinical trials for their value in improving patient care and
5 outcomes, as well as identifying potential novel signalling pathways involved in disease that may be
6 therapeutic targets.
7

8
9 In conclusion, BioHEART-CT is a study created in response to patients who have had heart attacks
10 and their frequent question from the cardiac catheter laboratory table- "Why me?". While it is
11 imperative that we measure and treat well-established risk factors for atherosclerosis, this approach
12 misses a substantial group of patients with CAD who are not identified as being at risk. We
13 hypothesise that major mechanisms for atherosclerosis remain to be discovered and can be
14 unravelled by comprehensive molecular characterisation, combined with powerful bioinformatic
15 analyses. BioHEART-CT's platforms and team are well positioned for discovering new markers and
16 mechanisms of disease, and to take these both back to the bench to unravel new biology, as well as
17 through to prospective clinical trial to test prognostic and clinical utility.
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Ethics and Dissemination

This study was approved by the Human Research Ethics Committee of the North Shore Local Health District, Sydney, Australia – approval number HREC/17/HAWKE/343, and has been registered as a clinical trial on the Australian New Zealand Clinical Trials Registry (ACTRN12618001322224).

Informed consent is obtained from participants prior to enrolment, and the data and samples are de-identified and managed entirely anonymously with the exception of the required information for follow-up phone calls. The most significant risk to the patients in this study is that of venesection, with a possible consequence of mild bruising or superficial infection. Participants can withdraw from the study at any time and this will not have any impact on their clinical care.

The results of this study will be published in peer-reviewed journals and presented at domestic and international scientific meetings. No identifiable information will be published.

Author's Contributions

KK and SV participated in study design, drafted the manuscript and coordinated manuscript revisions.

GF obtained the research funding and is the principal investigator of the study.

All other authors contributed to study design and revisions of the manuscript.

Author's Acknowledgements

The authors would also like to acknowledge in particular the extensive efforts of Luisa Osorio in supporting the BioHEART study. We would also like to extend thanks to the research coordinators and nurses for their hard work across all sites, as well as the nursing staff, radiologists and radiographers at the involved imaging facilities for their assistance. This project would not be possible without their support.

Funding Statement

GF is supported by NHMRC Practitioner Fellowship. SMG thanks the Parker Hughes Bequest for support. This study has received support from a combination of grants including from the Ramsay Teaching and Research Foundation, BioPlatforms Australia, the Vonwiller Foundation and Heart Research Australia.

Competing Interests Statement

None declared

References

- 1 Roth, G. A. *et al.* Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. *Journal of the American College of Cardiology* **70**, 1-25, doi:<https://doi.org/10.1016/j.jacc.2017.04.052> (2017).
- 2 Wang, H. *et al.* Global, regional, and national under-5 mortality, adult mortality, age-specific mortality, and life expectancy, 1970-2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet* **390**, 1084-1150, doi:10.1016/S0140-6736(17)31833-0 (2017).
- 3 *Australian Bureau of Statistics: Australia's leading causes of death*, <<http://www.abs.gov.au/>> (2016).
- 4 Welness, A. I. o. H. a. Australia's Health 2014.
- 5 Vernon, S. T. *et al.* Increasing proportion of ST elevation myocardial infarction patients with coronary atherosclerosis poorly explained by standard modifiable risk factors. *Eur J Prev Cardiol* **24**, 1824-1830, doi:10.1177/2047487317720287 (2017).
- 6 Mahmood, S. S., Levy, D., Vasan, R. S. & Wang, T. J. The Framingham Heart Study and the epidemiology of cardiovascular disease: a historical perspective. *Lancet* **383**, 999-1008, doi:10.1016/S0140-6736(13)61752-3 (2014).
- 7 Khot, U. N. *et al.* Prevalence of conventional risk factors in patients with coronary heart disease. *JAMA* **290**, 898-904, doi:10.1001/jama.290.7.898 (2003).
- 8 Wang, J. Y. *et al.* Cardiovascular Risk Factors and In-hospital Mortality in Acute Coronary Syndromes: Insights From the Canadian Global Registry of Acute Coronary Events. *Canadian Journal of Cardiology* **31**, 1455-1461, doi:10.1016/j.cjca.2015.04.007 (2015).
- 9 Vos, T. *et al.* Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet* **390**, 1211-1259, doi:10.1016/S0140-6736(17)32154-2 (2017).
- 10 The, C. D. C. Large-scale association analysis identifies new risk loci for coronary artery disease. *Nature genetics* **45**, 25-33, doi:10.1038/ng.2480 (2013).
- 11 Ridker, P. M. *et al.* Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *N Engl J Med* **377**, 1119-1131, doi:10.1056/NEJMoa1707914 (2017).
- 12 Harden, S. *et al.* The safe practice of CT coronary angiography in adult patients in UK imaging departments. *Clinical Radiology* **71**, 6 (2016).
- 13 Abbara, S. *et al.* SCCT guidelines for the performance and acquisition of coronary computed tomographic angiography: A report of the Society of Cardiovascular Computed Tomography Guidelines Committee. *Journal of Cardiovascular Computed Tomography* **10**, 435-449, doi:10.1016/j.jcct.2016.10.002 (2016).
- 14 Leipsic, J. *et al.* SCCT guidelines for the interpretation and reporting of coronary CT angiography: a report of the Society of Cardiovascular Computed Tomography Guidelines Committee. *J Cardiovasc Comput Tomogr* **8**, 342-358, doi:10.1016/j.jcct.2014.07.003 (2014).

- 1
2
3
4 15 Riedhammer, C., Halbritter, D. & Weissert, R. Peripheral Blood Mononuclear Cells: Isolation,
5 Freezing, Thawing, and Culture. *Methods in molecular biology (Clifton, N.J.)* **1304**, 53-61,
6 doi:10.1007/7651_2014_99 (2016).
7
- 8 16 Parker, B. L. *et al.* Multiplexed Temporal Quantification of the Exercise-regulated Plasma
9 Peptidome. *Mol Cell Proteomics* **16**, 2055-2068, doi:10.1074/mcp.RA117.000020 (2017).
10
- 11 17 Wang, T. J. *et al.* 2-Amino adipic acid is a biomarker for diabetes risk. *Journal of Clinical*
12 *Investigation* **123**, 4309+ (2013).
13
- 14 18 Roberts, L. D. *et al.* beta-Aminoisobutyric acid induces browning of white fat and hepatic beta-
15 oxidation and is inversely correlated with cardiometabolic risk factors. *Cell Metab* **19**, 96-108,
16 doi:10.1016/j.cmet.2013.12.003 (2014).
17
- 18 19 Turer, A. T. *et al.* Increases in myocardial workload induced by rapid atrial pacing trigger
19 alterations in global metabolism. *PLoS One* **9**, e99058, doi:10.1371/journal.pone.0099058
20 (2014).
21
- 22 20 Patsch, C. *et al.* Generation of vascular endothelial and smooth muscle cells from human
23 pluripotent stem cells. *Nat Cell Biol* **17**, 994-1003, doi:10.1038/ncb3205 (2015).
24
- 25 21 Hinson, J. T. *et al.* Integrative Analysis of PRKAG2 Cardiomyopathy iPS and Microtissue
26 Models Identifies AMPK as a Regulator of Metabolism, Survival, and Fibrosis. *Cell Rep* **17**,
27 3292-3304, doi:10.1016/j.celrep.2016.11.066 (2016).
28
- 29 22 Lomax, A. J. *et al.* Immunotherapy-induced sarcoidosis in patients with melanoma treated
30 with PD-1 checkpoint inhibitors: Case series and immunophenotypic analysis. *Int J Rheum*
31 *Dis* **20**, 1277-1285, doi:10.1111/1756-185X.13076 (2017).
32
- 33 23 Guzik, T. J., Skiba, D. S., Touyz, R. M. & Harrison, D. G. The role of infiltrating immune cells
34 in dysfunctional adipose tissue. *Cardiovasc Res* **113**, 1009-1023, doi:10.1093/cvr/cvx108
35 (2017).
36
- 37 24 Warren, C. R. *et al.* Induced Pluripotent Stem Cell Differentiation Enables Functional
38 Validation of GWAS Variants in Metabolic Disease. *Cell Stem Cell* **20**, 547-557 e547,
39 doi:10.1016/j.stem.2017.01.010 (2017).
40
- 41 25 Gensini, G. G. A more meaningful scoring system for determining the severity of coronary
42 heart disease. *American Journal of Cardiology* **51**, 606, doi:10.1016/S0002-9149(83)80105-2
43 (1983).
44
- 45 26 Tesche, C. *et al.* Coronary CT angiography derived morphological and functional quantitative
46 plaque markers correlated with invasive fractional flow reserve for detecting hemodynamically
47 significant stenosis. *Journal of Cardiovascular Computed Tomography* **10**, 199-206,
48 doi:10.1016/j.jcct.2016.03.002 (2016).
49
- 50 27 Vernon, S. T. *et al.* Utilizing state-of-the-art "omics" technology and bioinformatics to identify
51 new biological mechanisms and biomarkers for coronary artery disease. *Microcirculation* **0**,
52 e12488, doi:doi:10.1111/micc.12488.
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For peer review only

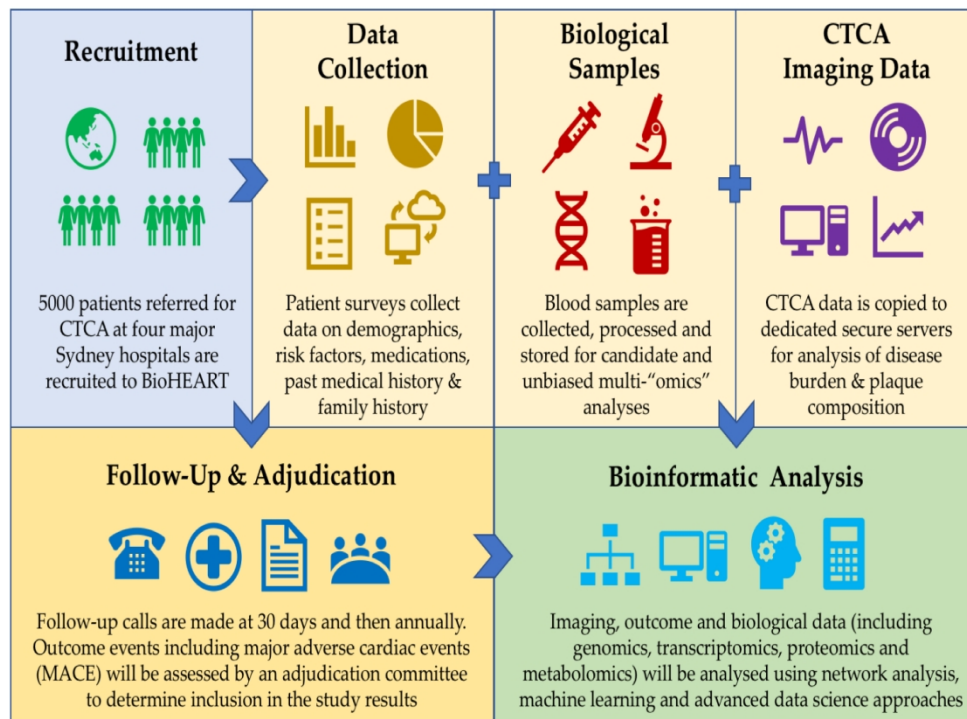


Figure 1: BioHEART-CT Study Design

359x269mm (110 x 110 DPI)

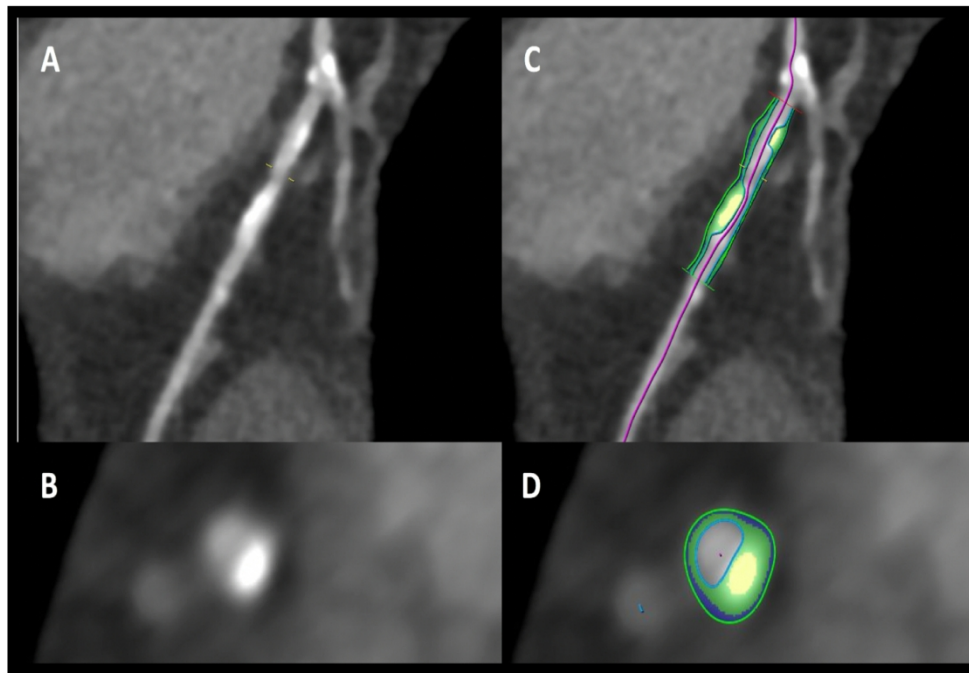


Figure 2: Example of semi-automated analysis of a complex plaque using syngo.via running on a Frontier system (Siemens Healthcare, Erlangen). Panels A & B show a coronary artery lesion with a large calcified component longitudinally and in cross-section. Panels C & D show classification of the calcific (yellow) and fibrous (green) components of this lesion

359x269mm (110 x 110 DPI)

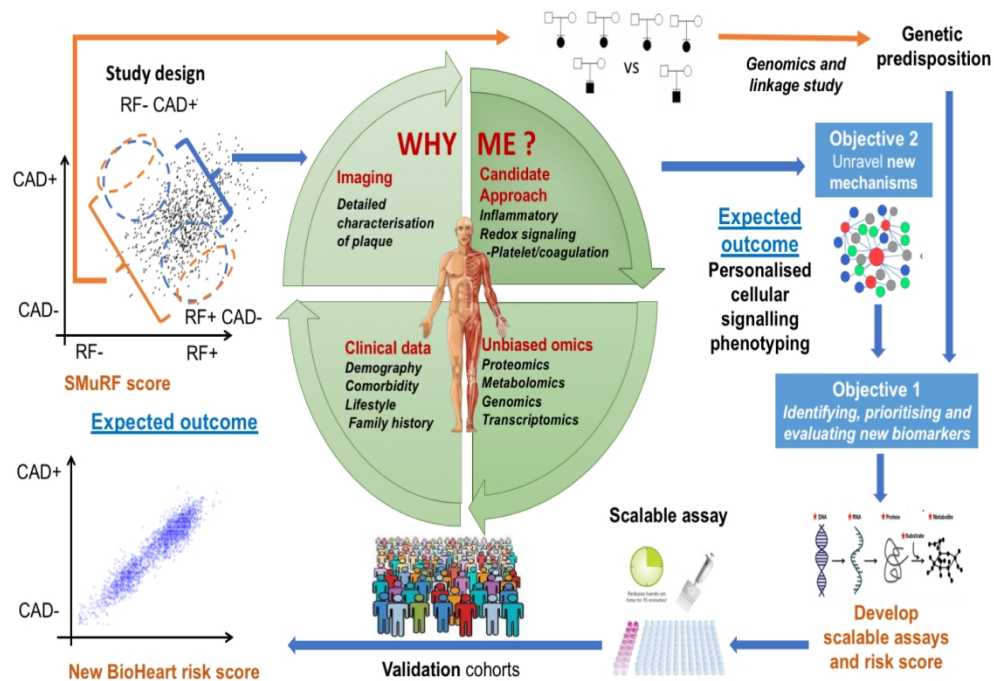


Figure 3: Overview of the Analytic Approach.

Precise phenotype characterisation including advanced plaque quantification and analysis of CTCA imaging and clinical data will be utilised in candidate and unbiased omics based assessments. Machine learning approaches will be applied to: identify new biomarkers, biological mechanisms of atherosclerosis, and new risk scores.

359x269mm (110 x 110 DPI)

BMJ Open

Biobanking for discovery of novel cardiovascular biomarkers using imaging-quantified disease burden: protocol for the longitudinal, prospective, BioHEART-CT cohort study

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2018-028649.R1
Article Type:	Protocol
Date Submitted by the Author:	28-Jun-2019
Complete List of Authors:	Kott, Katharine; University of Sydney, Sydney Medical School, Faculty of Medicine and Health Vernon, Stephen; University of Sydney, Sydney Medical School, Faculty of Medicine and Health Hansen, Thomas ; Kolling Institute of Medical Research Yu, Christine; Kolling Institute of Medical Research Bubb, Kristen ; University of Sydney, Northern Clinical School, Faculty of Medicine and Health Coffey, Sean; Royal North Shore Hospital, Kolling Institute and Department of Cardiology; University of Otago, Dunedin School of Medicine Sullivan, David; The University of Sydney Yang, Jean ; University of Sydney, School of Mathematics and Statistics O'Sullivan, John; University of Sydney, Sydney Medical School, Faculty of Medicine and Health Chow, Clara; The George Institute for Global Health and Westmead Hospital, Sydney Patel, Sanjay; University of Sydney, Sydney Medical School, Faculty of Medicine and Health Chong, James ; University of Sydney, Sydney Medical School, Faculty of Medicine and Health Celermajer, David; University of Sydney, Faculty of Medicine and Health; Royal Prince Alfred Hospital, Department of Cardiology Kritharides, Leonard; Concord Hospital, Cardiology Grieve, Stuart; University of Sydney, Sydney Medical School, Faculty of Medicine and Health Figtree, Gemma; University of Sydney, Faculty of Medicine and Health; Kolling Institute of Medical Research,
Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Patient-centred medicine, Radiology and imaging
Keywords:	Coronary heart disease < CARDIOLOGY, Cardiovascular imaging < RADIOLOGY & IMAGING, Ischaemic heart disease < CARDIOLOGY, Risk management < HEALTH SERVICES ADMINISTRATION & MANAGEMENT, MOLECULAR BIOLOGY, Clinical chemistry < PATHOLOGY

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



SCHOLARONE™
Manuscripts

1
2
3
4 1 Biobanking for discovery of novel cardiovascular
5
6
7 2 biomarkers using imaging-quantified disease burden:
8
9
10 3 protocol for the longitudinal, prospective, BioHEART-
11
12
13 4 CT cohort study
14
15

16 5 Katharine A. Kott*^{1,2}, Stephen T. Vernon*^{1,2}, Thomas Hansen¹, Christine Yu¹,
17 6 Kristen J. Bubb¹, Sean Coffey^{1,5}, David Sullivan^{2,3,6}, Jean Y. Yang^{3,4}, John F.
18 7 O'Sullivan^{2,3,9}, Clara K Chow^{2,10}, Sanjay Patel^{2,3,7,9}, James Chong^{2,10}, David S.
19 8 Celermajer^{2,7,9}, Leonard Kritharides^{2,11}, Stuart M. Grieve^{2,3,8}, Gemma A. Figtree^{1,2}

20
21
22
23 9 ¹Cardiothoracic and Vascular Health, Kolling Institute and Department of Cardiology, Royal North Shore Hospital,
24 10 Northern Sydney Local Health District, Australia

25 11 ²Sydney Medical School, Faculty of Medicine and Health, University of Sydney, Australia

26 12 ³Charles Perkins Centre, University of Sydney, Australia

27 13 ⁴School of Mathematics and Statistics, University of Sydney, Australia

28 14 ⁵Dunedin School of Medicine, University of Otago, New Zealand

29 15 ⁶Department of Biochemistry, Royal Prince Alfred Hospital, Sydney, Australia

30 16 ⁷Department of Cardiology, Royal Prince Alfred Hospital, Sydney, Australia

31 17 ⁸Department of Radiology, Royal Prince Alfred Hospital, Sydney, Australia

32 18 ⁹The Heart Research Institute, Sydney, Australia

33 19 ¹⁰Westmead Institute for Medical Research & Cardiology Department, Westmead Hospital, Sydney, Australia

34 20 ¹¹ANZAC Research Institute & Cardiology Department, Concord Repatriation General Hospital, Sydney, Australia

35 21 *The first two authors contributed equally to this publication
36 22

37
38
39 23 Corresponding author:

40 24 Professor Gemma Figtree MBBS DPhil (Oxon) FRACP FAHA

41
42 25 Cardiothoracic and Vascular Health, Level 12, Kolling Institute of Medical Research, Royal North Shore Hospital,
43 26 St Leonards, NSW, 2065, Australia. Email: gemma.figtree@sydney.edu.au.
44

45 27

46 28

47 29

48 30 Word Count: 3834

49
50
51
52
53 31
54
55
56
57
58
59
60

Abstract

Introduction: Coronary artery disease (CAD) persists as a major cause of morbidity and mortality worldwide despite intensive identification and treatment of traditional risk factors. Data emerging over the past decade shows a quarter of patients have disease in the absence of any known risk factor, and half have only one risk factor. Improvements in quantification and characterisation of coronary atherosclerosis by CT coronary angiography (CTCA) can provide quantitative measures of subclinical atherosclerosis - enhancing the power of unbiased “omics” studies to unravel the missing biology of personal susceptibility, identify new biomarkers for early diagnosis, and to suggest new targeted therapeutics.

Methods & Analysis: BioHEART-CT is a longitudinal, prospective cohort study, aiming to recruit 5000 adult patients undergoing clinically-indicated CTCA. After informed consent, patient data, blood samples and CTCA imaging data are recorded. Follow-up for all patients is conducted one month after recruitment, and then annually for the life of the study. CTCA data provides volumetric quantification of total calcified and non-calcified plaque, which will be assessed using established and novel scoring systems. Comprehensive molecular phenotyping will be performed using state-of-the-art genomics, metabolomics, proteomics, and immunophenotyping. Complex network and machine learning approaches will be applied to biological and clinical datasets to identify novel pathophysiological pathways and to prioritise new biomarkers. Discovery analysis will be performed in the first 1000 patients of BioHEART-CT, with validation analysis in the following 4000 patients. Outcome data will be utilised to build improved risk models for CAD.

Ethics & Dissemination: The study protocol has been approved by the human research ethics committee of North Shore Local Health District in Sydney, Australia. All findings will be published in peer-reviewed journals or at scientific conferences.

Registration: This study is registered with the Australia and New Zealand Clinical Trials Registry - ACTRN12618001322224.

1 Article Summary

2 Strengths and Limitations of this Study (max five bullet points)

- 3 - BioHEART-CT is a prospective, longitudinal cohort study assessing patients with suspected
4 CAD undergoing CTCA across multiple Australian centres.
- 5 - Quantitative measures of coronary atherosclerosis from CTCA datasets will be used in
6 conjunction with biological samples, improving the power to discover new mechanisms and
7 markers for CAD.
- 8 - Samples stratified by imaging-quantified disease severity will be analysed by both candidate
9 and unbiased “omics” approaches, utilising modern technologies and bioinformatic analysis
10 to discover new biomarkers using proteomics, metabolomics, lipidomics, transcriptomics,
11 genomics and immunophenotyping.
- 12 - Longitudinal outcome data will be used to build risk models which can incorporate both
13 traditional and novel risk factors and biomarkers.
- 14 - Limitations of the study include selection bias, as only patients with a clinical indication for
15 CTCA are included, and the geographic isolation of Australia, which may result in the need
16 for confirmation of results in multi-national studies.

1 Introduction

2 Cardiovascular disease has persisted as a major cause of human morbidity and mortality despite
3 continual improvements in preventative therapies. In 2015 cardiovascular disease accounted for
4 one-third of all deaths world-wide¹, and ischaemic heart disease (IHD) remains the leading cause of
5 years of life lost for high and middle sociodemographic groups multi-nationally². This large burden of
6 disease puts significant stress on the health systems of individual countries. In Australia,
7 cardiovascular disease remains the number one killer of Australians³, with local health costs
8 estimated to be \$7.7 billion AUD per annum⁴.

9 The standard modifiable cardiovascular risk factors (SMuRFs⁵) for atherosclerosis - hypertension,
10 diabetes mellitus, hyperlipidaemia and smoking - were identified in epidemiological studies in the
11 1960s⁶. The identification and subsequent efforts to target these risk factors at community and
12 primary care levels have led to a substantial reduction in mortality from cardiovascular disease¹.
13 However, substantial disease burden remains and importantly, individual variability in susceptibility
14 to these risk factors is considerable. Conditions such as impaired glucose tolerance and pre-
15 hypertension may also contribute to cardiovascular risk. While continuing our efforts to tackle
16 societal and modifiable risk factors, identifying undiscovered mechanisms that lead to the
17 development of atherosclerosis is critical. Such work will provide new biomarkers for early detection
18 of subclinical atherosclerosis and open avenues for improved preventative and therapeutic
19 strategies that may be relevant to those with and without known risk factors.

20 The importance of new detection mechanisms for subclinical atherosclerosis is highlighted by
21 interrogating the “fine print” of clinical studies, including information not usually presented in the
22 main tables of manuscripts. Although data regarding the percentage of patients suffering
23 myocardial infarction despite having no SMuRFs is often omitted, a large meta-analysis found that
24 >50% of women and >60% of men presenting with their first ACS had 0 or 1 SMuRF⁷, and similar
25 proportions of SMuRFless patients have been identified in ACS cohort studies^{5,8}. In a recent single
26 centre Australian study, we showed that the proportion of patients presenting with ST-elevation
27 myocardial infarction with 0 SMuRFs has risen from 11% to 27% over an 8 year period⁵. These
28 patients have developed extensive CAD without having any of the red flags which would allow
29 doctors to identify their risk of disease and intervene early. Although this is a relatively small piece of
30 the overall IHD pie, the global burden of CAD is immense with an estimated prevalence of 470
31 million⁹, which makes this 25-30% SMuRFless patient population important from a public health
32 perspective. It is sobering to think that we have made no clear advances that allow us to identify risk
33 and subclinical disease in this subgroup. Indeed, we also have limited information regarding how
34 these patients respond to traditional secondary preventative strategies, and whether their long term
35 outcomes and disease progression differs from those with traditional modifiable risk factors.

36 Further evidence that novel biological mechanisms contribute substantially to atherosclerosis can be
37 seen in results from large scale genome-wide association studies which have reported that 66% of
38 the identified genes conveying heritable cardiovascular risk are not associated with the traditional
39 risk factors¹⁰. Many of these genes were related to inflammatory processes, and studies such as the
40 CANTOS trial¹¹ confirm the importance of inflammation in CAD. However, some of these important
41 genes have not yet been well characterised, and are not yet associated with any known pathway in
42 atherosclerosis and CAD, highlighting the importance of ongoing discovery work.

43 To address this gap in our understanding of CAD pathophysiology, we have designed a unique cohort
44 study with biobanked samples to facilitate investigation of novel cardiac risk factors and biomarkers.

1 This biobank includes advanced, quantitative imaging measurements of coronary artery
2 atherosclerosis to allow for accurate phenotyping of the patient groups. This overcomes a
3 weaknesses of previous genomics studies that have relied on the presence or absence of a coronary
4 artery event to classify individuals as diseased or as a control, improving the statistical power of the
5 subsequent analysis. The advanced imaging dataset and clinical data including baseline and follow-
6 up are integrated with the collection of a broad range of biological samples for discovery work,
7 utilising state-of-the-art molecular phenotyping platforms and computational bioinformatics.

8 Objectives

9 The primary objectives of the BioHEART-CT study are:

- 10 1. To identify new biomarkers to assist in early CAD risk identification and stratification;
- 11 2. To identify new mechanistic pathways that may be targeted with novel therapeutic
12 strategies to abrogate CAD risk.

13 The secondary objectives of the BioHEART-CT study include the following:

- 14 1. To determine the predictive value of a modified CTCA scoring system for CAD risk which
15 incorporates plaque composition data;
- 16 2. To identify new risk factors for CAD based on integration of clinical information profiles,
17 CTCA results and clinical outcomes;
- 18 3. To develop a novel risk scoring system incorporating clinical risk factors, novel
19 biomarkers and CTCA scores to more accurately predict CVD events.

20 Methods and Analysis

21 Patient and Public Involvement

22 The initial concept for the BioHEART-CT study was prompted by patients who had no known
23 cardiovascular risk factors who presented with ACS and wanted to know “why me?” The ensuing
24 discussions with the patients and their families reinforced the public interest in research in this area.
25 Informal consultation with patient groups through cardiovascular non-profit organisations helped
26 build the initial framework for the study, and patient feedback regarding the recruitment process
27 was sought and was positive in nature. When results of interest to the general public are available,
28 summaries will be posted to the Australia and New Zealand Clinical Trials Registry. Information
29 about this registry is included on the patient information sheet.

30 Study Type

31 BioHEART-CT is a multicentre, prospective cohort study of patients with suspected CAD, which brings
32 together detailed clinical information with advanced imaging and molecular phenotyping.
33 Recruitment commenced in 2017 and is ongoing, currently with expansion into a multi-centre study.

34 Study Population

35 5000 patients will be recruited from participating hospitals’ and associated imaging sites. All
36 recruitment centres are large tertiary hospitals within the city of Sydney, Australia.

37 Inclusion criteria:

- 38 - Patients who have been referred for investigation of suspected CAD
- 39 - Age 18 years or older

- 1
2
3 1 - Willing and able to provide informed consent
4

5 2 Exclusion criteria:

- 6
7 3 - Patients highly dependent on medical care who are unable to provide informed consent
8 4 - Patients unwilling or unable to participate in ongoing follow-up
9

10 5 **Study Protocol**

11 6 **Screening and Enrolment**

12
13 7 Eligible patients having a CTCA are invited to participate in the study. Eligibility is confirmed
14 8 according to the inclusion and exclusion criteria, the study is explained, and informed consent
15 9 obtained (Figure 1).
16
17
18

19 10 **Patient Data Collection**

20 11 The baseline questionnaire is completed by research staff conducting a face-to-face patient
21 12 interview. Data obtained includes the following information: demographic data including smoking
22 13 and alcohol intake, past medical history, relevant family history, medication history, history of
23 14 cardiac symptoms and indication for CTCA. Data is entered into secure, encrypted databases in a de-
24 15 identified format. After recruitment all patients are assigned a unique study number and all samples,
25 16 imaging data and demographic information is identified by this number. The master list including
26 17 identifiable data is securely stored on an encrypted server and is available only to senior research
27 18 staff. Relevant details are made available to research staff for follow-up purposes.
28
29
30

31 19 **Follow-Up & Outcome Adjudication**

32 20 Participants are contacted by phone at approximately 30 days and then annually to determine if any
33 21 events have occurred. Information recorded for any potential outcome event includes the acuity,
34 22 need for hospitalisation and indication for admission, hospital attended, treating physician, date of
35 23 admission and details of the events. New medical or surgical diagnoses since last contact are also
36 24 recorded. Participants in BioHEART also consent to review of their electronic medical records and
37 25 data linkage through the Centre for Health Record Linkage (CHeReL), which will be used for
38 26 independent medical record reviews.
39
40
41

42 27 Significant outcome events that trigger a file review to assess for major adverse cardiovascular
43 28 events (MACE) or secondary outcomes are the following:

- 44
45 29 - Unstable angina requiring hospitalisation
46 30 - Heart failure requiring hospitalisation
47 31 - Acute myocardial infarction
48 32 - Coronary angiogram with or without percutaneous intervention
49 33 - Cardiac bypass graft surgery
50 34 - Aortic or mitral valve surgery
51 35 - Cerebrovascular accident / transient ischaemic attack
52 36 - All-cause mortality
53
54

55 37 All medical records will be reviewed by an independent adjudication committee to determine if an
56 38 event should be included in the study results. Detailed criteria for the individual diagnoses included
57 39 in the outcomes must be met.
58
59
60

1 MACE is defined as cardiovascular death, non-fatal myocardial infarction or non-fatal stroke.
2 Exploratory outcomes include revascularisation, unstable angina or heart failure requiring
3 hospitalisation.

4 CTCA Data Acquisition

5 CTCA scans are obtained on 256 slice scanner, with reconstructions created using appropriate
6 software for the individual machine. Each study is to be protocolled by a radiologist with at least
7 level two accreditation (RANZCR/RANZCP). All radiographers are trained in acquisition and workup
8 of CTCA. Oral metoprolol, or ivabradine if beta blockers are contraindicated, are given to optimise
9 the heart rate if required. Dosing is adjusted by clinical staff according to baseline heart rate and
10 bodyweight. Oral nitroglycerine (600-800 micrograms) is given immediately prior to the scan, and
11 iodinated contrast is injected intravenously. Prospective studies are performed if the heart rate is
12 sufficiently controlled, otherwise retrospective acquisition is used. Average dose is minimised at all
13 participating sites in line with current recommendations^{12,13}.

14 CTCA Imaging Analysis

15 CT data is exported as thin DICOM data and is stored securely in a de-identified manner. Data is later
16 analysed using a dedicated workstation to obtain the primary clinical scores at each site. CTCAs are
17 analysed by standard anatomical arterial region as per the 17-segment model outlined by the
18 Society of Cardiovascular Computed Tomography¹⁴. Each segment is scored according to degree of
19 stenosis and composition of plaque (calcified, soft and mixed). The segmental scores are aggregated
20 into a modified GENSINI score (Figure 2) which represents the total amount of calcified and non-
21 calcified plaque present. Calcium scores are generated using the scanner-associated software by the
22 Agaston method. Total raw calcium scores, raw calcium scores for each vessel, and age and sex-
23 matched calcium percentage scores are recorded.

24 Further analysis will be performed at a central core laboratory using a Frontier System (syngo.via,
25 Siemens Healthcare, Erlangen), employing a semi-automated soft plaque algorithm (Figure 3).

26 Biological Sample Collection

27 A 20-30mL blood sample is taken from an in-situ intravenous cannula, or if this is not available, by
28 venesection, prior to CTCA and drug administration. Blood is collected into EDTA, serum and citrate
29 tubes and stored at 4°C until processing. A lithium heparin tube is collected and processed
30 immediately to extract peripheral blood mononuclear cells (PBMCs) from the buffy coat.

31 Sample Processing & Storage

32 Blood samples are processed by hospital pathology scientists experienced in laboratory procedures.
33 Briefly, whole blood is set aside, then tubes are centrifuged at 1861 x g for 15 minutes at 4°C prior to
34 aliquoting 500uL samples of serum, EDTA plasma, whole blood, buffy coat and erythrocytes. The
35 citrate tube is centrifuged at 1861 x g for a second 15 minutes at 4°C, after which citrate-plasma
36 samples are aliquoted. All aliquoted samples are frozen and stored at -80°C until analysis.

37 For PBMC isolation a standard gradient-separation protocol is used¹⁵. Briefly, blood from the lithium
38 heparin tube is diluted 1:1 with Hanks' Balanced Salt Solution (HBSS). Diluted whole blood is then
39 layered on top of Ficoll-Paque Plus and centrifuged (22°C, 1460 x g no brake, 20 min) . The buffy coat
40 layer containing PBMCs is then further purified with two wash steps in HBSS using repeat
41 centrifugation as above. PBMCs are then plated at a density of ~2.5 x 10⁴ cells/cm² into 0.1% gelatin-
42 coated flasks containing endothelial cell growth medium with 2% fetal bovine serum (EGM2

bulletkit, Lonza, Australia) and cultured in standard conditions for up to 21 days with regular monitoring for the spontaneous growth of Endothelial Progenitor Cells (EPCs). Excess PBMCs are immediately frozen in bovine serum (heat-inactivated, Gibco, Australia) containing 10% dimethyl sulfoxide and stored in liquid nitrogen.

Sample Analysis

Samples will be analysed by a variety of methods with the intent of identifying candidate biomarkers and novel metabolites involved in atherosclerosis pathophysiology. There will be two principal approaches used to identify these factors. Table 1 provides a summary of all variables collected and planned assays.

TABLE 1 Summary of variables and planned assays

Clinical Variables	Disease Quantitation Variables	Established Markers	Unbiased -omics Approaches	Candidate Approaches
Demographics Standard modifiable cardiovascular risk factors (SMuRFs) - Hypertension - Hyperlipidaemia - Diabetes - Smoking Other medical history BMI Family history of IHD Current medications History of cardiac symptoms	Coronary artery calcium scores (CACS) GENSINI score Modified GENSINI score (see figure 2) Frontier System semi-automated plaque analysis (syngo.via, Siemens Healthcare, Erlangen)	Cardiac - Troponin - NT-BNP Inflammatory - CRP - VCAM-1 - ICAM-1 - IL-6	Metabolomics Proteomics Lipidomics Transcriptomics Genomics Immunophenotyping	Redox signalling dysregulation Endothelial cell signalling dysregulation Soluble factors released by inflammatory plaque Apoptosis signalling Mitochondrial function Angiogenesis potential Disorders of overall coagulation Soluble platelet factors Alpha-gal antibodies Heavy metal toxicities Galectin-3

First is a candidate approach, investigating currently identified factors that are thought to be involved in human biological pathways that could predispose to atherosclerosis. These factors will be assessed within the cohort by specific assays that relate to the biological pathways involved. The candidate areas that have been identified for initial assessment are: soluble factors released by inflammatory signalling within the atherosclerotic plaque, dysregulated redox signalling within the endothelium, disorders of coagulation which lead to hypercoagulable states and/or hypofibrinolysis, soluble platelet factors which indicate platelet dysfunction, and dysregulated signalling within

1
2
3 1 endothelial cells as evidenced by assessment of cultured EPCs derived from individual patient
4 2 samples. Individual assays have been designed to assess each candidate marker or pathway.

6 3 The second approach aims to investigate atherosclerosis biology in an unbiased manner by using
7 4 “omics” techniques. These assessments will utilise metabolomics, proteomics, transcriptomics,
8 5 genomics including SNP arrays and immunophenotyping techniques. The cells utilised for
9 6 transcriptomics will be patient-derived EPCs and PBMCs.

11 7 Proteomics for the cohort will be assessed utilising a clinical proteomics platform¹⁶ which will give a
12 8 complete description of the human peptidome in relevant samples, allowing for identification of
13 9 novel or common factors worthy of further investigation. Metabolomics and lipidomics will be
14 10 performed utilising six liquid chromatography-mass spectrometry platforms which will allow for
15 11 identification and quantitation of a broad spectrum of metabolites, including amino acids,
16 12 nucleotides, neurotransmitters and lipid subtypes¹⁷⁻²¹.

19 13 Immunophenotyping will be performed on PBMC samples via mass spectrometry time of flight
20 14 (CyTOF) which allows for detailed leucocyte phenotyping on an individual basis²². These profiles can
21 15 be associated with serum levels of established markers of inflammation such as VCAM-1, ICAM-1
22 16 and IL-6, as well as correlated with the balance of M1 and M2 phenotype monocytes which have a
23 17 well established role in atherosclerosis²³.

26 18 Transcriptomic and genomic assays will be utilised on subsets of the cohort of particular interest,
27 19 who for example may display particular resistance or vulnerability to CAD based on traditional risk
28 20 factor profile and disease burden. Whole transcriptome and RNA sequencing will be performed with
29 21 ribosomal depletion, and sequencing of the resulting reads and counting will be based on GENCODE.
30 22 Whole genome profiling will be performed on individuals with extreme athero-susceptible, or
31 23 athero-resilient phenotype, allowing for unbiased discovery of genetic variants that may be causally
32 24 associated with the extreme phenotypes²⁴.

35 25 Data Analysis Plan

37 26 Statistical machine learning approaches will be applied to these biological and clinical datasets, to
38 27 identify novel pathophysiological pathways, and to prioritise new markers based on likely causal
39 28 roles. Discovery analysis will be performed in the first 1000 patients of BioHEART-CT, with validation
40 29 in the following 4000 patients recruited, and in international collaborators studies (Figure 4). Sample
41 30 size planning for developing classifiers using high dimensional data is based on NIH Biometric online
42 31 calculator assuming 1.3 fold standardised change, 50,000 molecular variables, and balanced extreme
43 32 groups.

46 33 Biomarker and risk factor data analysis will depend on the specifics of the assay or investigation
47 34 performed. Generally, continuous variables will be presented as means (with standard deviations) or
48 35 medians (with inter-quartile ranges), categorical variables as proportions (%) with chi-squared or
49 36 Fisher’s exact test used to determine differences between groups. Hazard ratios for a one standard
50 37 deviation increase in a biomarker, after log transformation to remove effects of outliers, on MACE
51 38 will be obtained from weighted Cox regression models. All p-values reported will be two-sided, with
52 39 the 5% threshold used to determine significance.

55 40 For testing the prognostic value of prioritised biomarkers, and assuming a C-statistic of 0.75 for
56 41 traditional risk factors, we estimate that we will require 705 participants to detect a C-statistic of
57 42 0.85 with $p=0.05$, power of 80% and an event rate of 20%. Models developed with different sets of
58 43 variables and biomarkers and C-statistics for one year risk will be determined and compared. The

1
2
3 1 model's ability to discriminate and reclassify one year risk will be assessed using the integrated
4 2 discrimination index and the net reclassification improvement.
5

6 7 3 Discussion 8

9 4 Three main advances have been applied in the design of BioHEART-CT that provide strategic
10 5 advantage compared to previous studies which also used unbiased approaches to discover novel
11 6 mechanisms and markers of atherosclerosis. First, this study includes quantification and
12 7 characterisation of plaque (and the absence of plaque) by CT coronary angiography and advanced
13 8 imaging algorithms, improving the power of the study compared to those that relied on CAD-related
14 9 clinical events. Second, this study integrates high through-put, state-of-the-art multi-omic molecular
15 10 phenotyping with comprehensive clinical data and follow up. And third, the study brings together a
16 11 global team of biologists and bioinformaticians with complementary approaches and perspectives to
17 12 enhance the likelihood of new discoveries.

18 13 In previous CAD studies, healthy controls were often defined as patients who had not had a cardiac
19 14 event, or who had non-obstructive atherosclerosis on invasive coronary angiography which has
20 15 limited utility in identifying extraluminal plaque. As CAD often remains silent until it is very
21 16 advanced, those without a myocardial infarction could have quite extensive disease and still be
22 17 considered a "healthy" control. In BioHEART-CT, we have the capability to use our CTCA subgroup to
23 18 more precisely phenotype subjects, allowing the accurate creation of a group of healthy controls
24 19 which truly have no atherosclerotic disease present in their coronary arteries. This group of healthy
25 20 patients can be used as a comparator for those with various stages of disease, reducing confounding
26 21 factors and enhancing the capacity to detect real differences in biology.

27 22 Additionally, CTCA phenotyping can be utilised to analyse plaque composition in detail. Using scoring
28 23 systems such as GENSINI²⁵, severity scores can be generated for each individual. We have also
29 24 developed a modified GENSINI score as outlined above, which gives different weight to the presence
30 25 of older, calcified plaque and newer, more biologically active non-calcified plaque. Automated
31 26 detection of soft plaque shows promise as a scalable, reproducible method to add value to existing
32 27 CTCA analysis²⁶. In identifying patients with high levels of active atherosclerotic plaque, we can
33 28 create groups enriched for this biological feature, with and without traditional risk factors. This will
34 29 better enable us to detect novel mechanisms of CAD in those with unexplained susceptibility.

35 30 Our unbiased approach to cardiovascular risk factor discovery, applying the advantages of high
36 31 throughput multi-omics platforms, improved quantification of plaque and its characteristics, and
37 32 advanced statistical bioinformatics, provides a high chance of discovering previously unidentified
38 33 mechanisms and markers of disease. We have recently discussed the advantages of integrating
39 34 multi-omics over isolated genomics studies for the complex disease process of atherosclerosis²⁷, and
40 35 this will be particularly beneficial in such well-phenotyped groups.

41 36 Molecular markers found to be associated with atherosclerosis may be direct drivers of disease, or
42 37 result from the plaque and dysregulated vascular physiology. The closer the marker is to the
43 38 underlying biology, the more likely it will be to be useful in clinical practice, transforming our ability
44 39 to identify early vascular disease and susceptibility above and beyond traditional risk calculators. We
45 40 are well placed to take any markers and their surrounding pathways into preclinical cellular and
46 41 animal models to determine causality and/or mechanisms for association. This will help prioritise
47 42 markers for testing in prospective clinical trials for their value in improving patient care and
48 43 outcomes, as well as identifying potential novel signalling pathways involved in disease that may be
49 44 therapeutic targets.

1
2
3 1 In conclusion, BioHEART-CT is a study created in response to patients who have had heart attacks
4 2 and their frequent question from the cardiac catheter laboratory table- “Why me?”. While it is
5 3 imperative that we measure and treat well-established risk factors for atherosclerosis, this approach
6 4 misses a substantial group of patients with CAD who are not identified as being at risk. We
7 5 hypothesise that major mechanisms for atherosclerosis remain to be discovered and can be
8 6 unravelled by comprehensive molecular characterisation, combined with powerful bioinformatic
9 7 analyses. BioHEART-CT’s platforms and team are well positioned for discovering new markers and
10 8 mechanisms of disease, and to take these both back to the bench to unravel new biology, as well as
11 9 through to prospective clinical trial to test prognostic and clinical utility.

10 Ethics and Dissemination

11 This study was approved by the Human Research Ethics Committee of the North Shore Local Health
12 District, Sydney, Australia – approval number HREC/17/HAWKE/343, and has been registered as a
13 clinical trial on the Australian New Zealand Clinical Trials Registry (ACTRN12618001322224).

14 Informed consent is obtained from participants prior to enrolment, and the data and samples are
15 de-identified and managed entirely anonymously with the exception of the required information for
16 follow-up phone calls. The most significant risk to the patients in this study is that of venesection,
17 with a possible consequence of mild bruising or superficial infection. Participants can withdraw from
18 the study at any time and this will not have any impact on their clinical care.

19 The results of this study will be published in peer-reviewed journals and presented at domestic and
20 international scientific meetings. No identifiable information will be published.

21 Author’s Contributions

22 KK and SV participated in study design, drafted the manuscript and coordinated manuscript
23 revisions.

24 GF obtained the research funding and is the principal investigator of the study.

25 All other authors (TH, CY, KB, SC, DS, JY, JO, CC, SP, JC, DS, LK, SG) contributed to study design and
26 revisions of the manuscript.

27 Author’s Acknowledgements

28 The authors would also like to acknowledge in particular the extensive efforts of Luisa Osorio in
29 supporting the BioHEART study. We would like to thank the patients and their families who advised
30 us on the importance of the research and the basic study design, and to all the patients who have
31 participated in the trial. We would also like to extend thanks to the research coordinators and nurses
32 for their hard work across all sites, as well as the nursing staff, radiologists and radiographers at the
33 involved imaging facilities for their assistance. This project would not be possible without their
34 support. We would also like to acknowledge the Vonwiller Foundation for their support and advice
35 on this project.

36 Funding Statement

37 GF is supported by NHMRC Practitioner Fellowship. SMG thanks the Parker Hughes Bequest for
38 support. This study has received support from a combination of grants including from the Ramsay

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1 Teaching and Research Foundation, BioPlatforms Australia, the Vonwiller Foundation and Heart
2 Research Australia.

3 **Competing Interests Statement**

4 None declared

5

6

For peer review only

References

- 1 Roth, G. A. *et al.* Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. *Journal of the American College of Cardiology* **70**, 1-25, doi:<https://doi.org/10.1016/j.jacc.2017.04.052> (2017).
- 2 Wang, H. *et al.* Global, regional, and national under-5 mortality, adult mortality, age-specific mortality, and life expectancy, 1970–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet* **390**, 1084-1150, doi:10.1016/S0140-6736(17)31833-0 (2017).
- 3 *Australian Bureau of Statistics: Australia's leading causes of death*, <<http://www.abs.gov.au/>> (2016).
- 4 Welness, A. I. o. H. a. Australia's Health 2014.
- 5 Vernon, S. T. *et al.* Increasing proportion of ST elevation myocardial infarction patients with coronary atherosclerosis poorly explained by standard modifiable risk factors. *Eur J Prev Cardiol* **24**, 1824-1830, doi:10.1177/2047487317720287 (2017).
- 6 Mahmood, S. S., Levy, D., Vasan, R. S. & Wang, T. J. The Framingham Heart Study and the epidemiology of cardiovascular disease: a historical perspective. *Lancet* **383**, 999-1008, doi:10.1016/S0140-6736(13)61752-3 (2014).
- 7 Khot, U. N. *et al.* Prevalence of conventional risk factors in patients with coronary heart disease. *JAMA* **290**, 898-904, doi:10.1001/jama.290.7.898 (2003).
- 8 Wang, J. Y. *et al.* Cardiovascular Risk Factors and In-hospital Mortality in Acute Coronary Syndromes: Insights From the Canadian Global Registry of Acute Coronary Events. *Canadian Journal of Cardiology* **31**, 1455-1461, doi:10.1016/j.cjca.2015.04.007 (2015).
- 9 Vos, T. *et al.* Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet* **390**, 1211-1259, doi:10.1016/S0140-6736(17)32154-2 (2017).
- 10 The, C. D. C. Large-scale association analysis identifies new risk loci for coronary artery disease. *Nature genetics* **45**, 25-33, doi:10.1038/ng.2480 (2013).
- 11 Ridker, P. M. *et al.* Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *N Engl J Med* **377**, 1119-1131, doi:10.1056/NEJMoa1707914 (2017).
- 12 Harden, S. *et al.* The safe practice of CT coronary angiography in adult patients in UK imaging departments. *Clinical Radiology* **71**, 6 (2016).
- 13 Abbara, S. *et al.* SCCT guidelines for the performance and acquisition of coronary computed tomographic angiography: A report of the Society of Cardiovascular Computed Tomography Guidelines Committee. *Journal of Cardiovascular Computed Tomography* **10**, 435-449, doi:10.1016/j.jcct.2016.10.002 (2016).
- 14 Leipsic, J. *et al.* SCCT guidelines for the interpretation and reporting of coronary CT angiography: a report of the Society of Cardiovascular Computed Tomography Guidelines Committee. *J Cardiovasc Comput Tomogr* **8**, 342-358, doi:10.1016/j.jcct.2014.07.003 (2014).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

- 1 15 Riedhammer, C., Halbritter, D. & Weissert, R. Peripheral Blood Mononuclear Cells: Isolation, Freezing, Thawing, and Culture. *Methods in molecular biology (Clifton, N.J.)* **1304**, 53-61, doi:10.1007/7651_2014_99 (2016).
- 2 16 Parker, B. L. *et al.* Multiplexed Temporal Quantification of the Exercise-regulated Plasma Peptidome. *Mol Cell Proteomics* **16**, 2055-2068, doi:10.1074/mcp.RA117.000020 (2017).
- 3 17 Wang, T. J. *et al.* 2-Aminoadipic acid is a biomarker for diabetes risk. *Journal of Clinical Investigation* **123**, 4309+ (2013).
- 4 18 Roberts, L. D. *et al.* beta-Aminoisobutyric acid induces browning of white fat and hepatic beta-oxidation and is inversely correlated with cardiometabolic risk factors. *Cell Metab* **19**, 96-108, doi:10.1016/j.cmet.2013.12.003 (2014).
- 5 19 Turer, A. T. *et al.* Increases in myocardial workload induced by rapid atrial pacing trigger alterations in global metabolism. *PLoS One* **9**, e99058, doi:10.1371/journal.pone.0099058 (2014).
- 6 20 Patsch, C. *et al.* Generation of vascular endothelial and smooth muscle cells from human pluripotent stem cells. *Nat Cell Biol* **17**, 994-1003, doi:10.1038/ncb3205 (2015).
- 7 21 Hinson, J. T. *et al.* Integrative Analysis of PRKAG2 Cardiomyopathy iPS and Microtissue Models Identifies AMPK as a Regulator of Metabolism, Survival, and Fibrosis. *Cell Rep* **17**, 3292-3304, doi:10.1016/j.celrep.2016.11.066 (2016).
- 8 22 Lomax, A. J. *et al.* Immunotherapy-induced sarcoidosis in patients with melanoma treated with PD-1 checkpoint inhibitors: Case series and immunophenotypic analysis. *Int J Rheum Dis* **20**, 1277-1285, doi:10.1111/1756-185X.13076 (2017).
- 9 23 Guzik, T. J., Skiba, D. S., Touyz, R. M. & Harrison, D. G. The role of infiltrating immune cells in dysfunctional adipose tissue. *Cardiovasc Res* **113**, 1009-1023, doi:10.1093/cvr/cvx108 (2017).
- 10 24 Warren, C. R. *et al.* Induced Pluripotent Stem Cell Differentiation Enables Functional Validation of GWAS Variants in Metabolic Disease. *Cell Stem Cell* **20**, 547-557 e547, doi:10.1016/j.stem.2017.01.010 (2017).
- 11 25 Gensini, G. G. A more meaningful scoring system for determining the severity of coronary heart disease. *American Journal of Cardiology* **51**, 606, doi:10.1016/S0002-9149(83)80105-2 (1983).
- 12 26 Tesche, C. *et al.* Coronary CT angiography derived morphological and functional quantitative plaque markers correlated with invasive fractional flow reserve for detecting hemodynamically significant stenosis. *Journal of Cardiovascular Computed Tomography* **10**, 199-206, doi:10.1016/j.jcct.2016.03.002 (2016).
- 13 27 Vernon, S. T. *et al.* Utilizing state-of-the-art "omics" technology and bioinformatics to identify new biological mechanisms and biomarkers for coronary artery disease. *Microcirculation* **0**, e12488, doi:doi:10.1111/micc.12488.

1
2
3
4 1
5
6
7
8 2
9 3
10
11 4
12
13 5
14
15 6
16
17 7
18
19 8
20
21 9
22 10
23
24 11
25
26 12
27
28 13
29
30 14
31
32 15
33
34 16
35
36 17
37
38 18
39
40 19
41
42 20
43
44 21
45
46 22
47
48 23
49
50
51
52
53
54
55
56
57
58
59
60

For peer review only

Figure Legends

Figure 1: BioHEART-CT Study Design

Figure 2: Semi-quantitative plaque analysis incorporating the established GENSINI scoring system²⁵ and adding an additional multiplier for plaque composition to create a modified GENSINI score.

Figure 3: Example of semi-automated analysis of a complex plaque using syngo.via running on a Frontier system (Siemens Healthcare, Erlangen). Panels A & B show a coronary artery lesion with a large calcified component longitudinally and in cross-section. Panels C & D show classification of the calcific (yellow) and fibrous (green) components of this lesion.

Figure 4: Overview of the Analytic Approach.

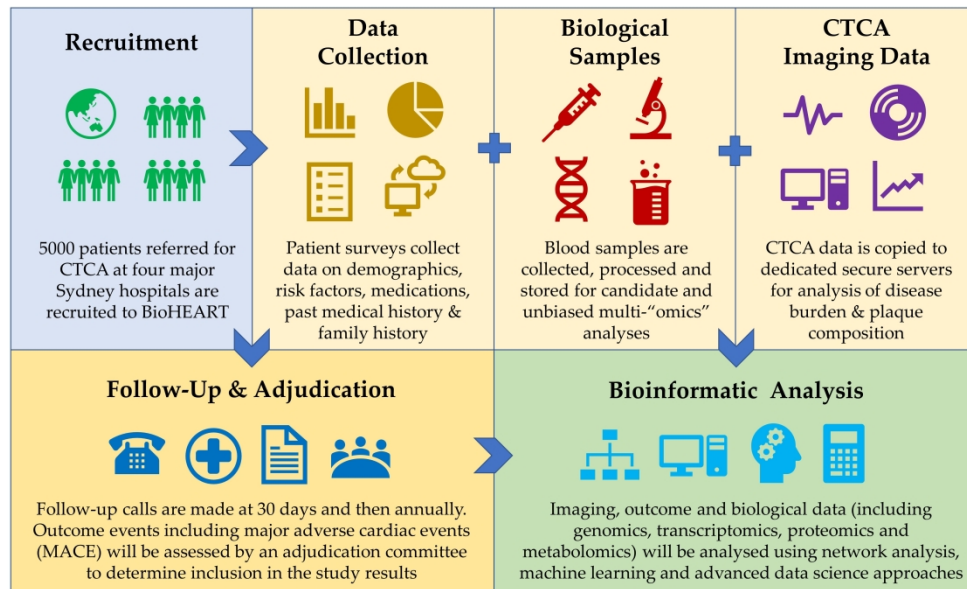


Figure 1: BioHEART-CT Study Design

358x220mm (300 x 300 DPI)

Stenosis Location	Multiplication Factor – Right Dominant (Left Dominant)		Percent Stenosis	Score
RCA			0	0
- Proximal / Mid / Distal /	x1		1-25%	1
- PDA	x1		26-50%	2
- PLB	x0.5		51-75%	4
LMCA	x5		76-90%	8
LAD		91-99%	16	
- Proximal	x2.5	100%	32	
- Mid	x1.5			
- Distal	x1.0			
- D1	x1.0			
- D2	x0.5			
LCx				
- Proximal	x2.5 (x3.5)			
- Distal	x1 (x2)			
- OM1	x1			
- OM2	x0.5			
- (Left PDA)	(x1)			
- (Left PLB)	(x0.5)			
Ramus	x1			
		Plaque Composition	Multiplication Factor	
		Calcified	x1	
		Mixed	x2	
		Non-calcified (soft)	x3	

Figure 2: Semi-quantitative plaque analysis incorporating the established GENSINI scoring system²⁵ and adding an additional multiplier for plaque composition to create a modified GENSINI score.

210x148mm (300 x 300 DPI)

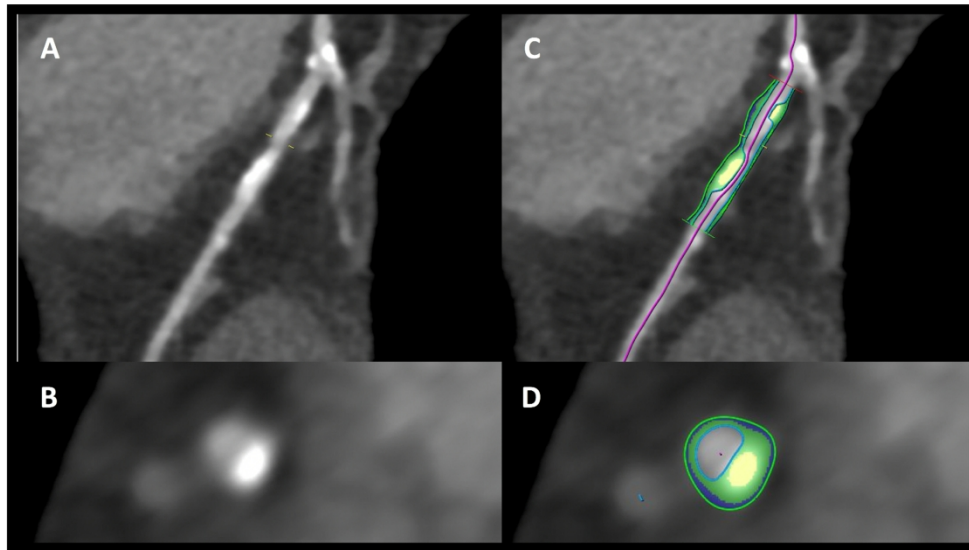


Figure 3: Example of semi-automated analysis of a complex plaque using syngo.via running on a Frontier system (Siemens Healthcare, Erlangen). Panels A & B show a coronary artery lesion with a large calcified component longitudinally and in cross-section. Panels C & D show classification of the calcific (yellow) and fibrous (green) components of this lesion.

358x220mm (300 x 300 DPI)

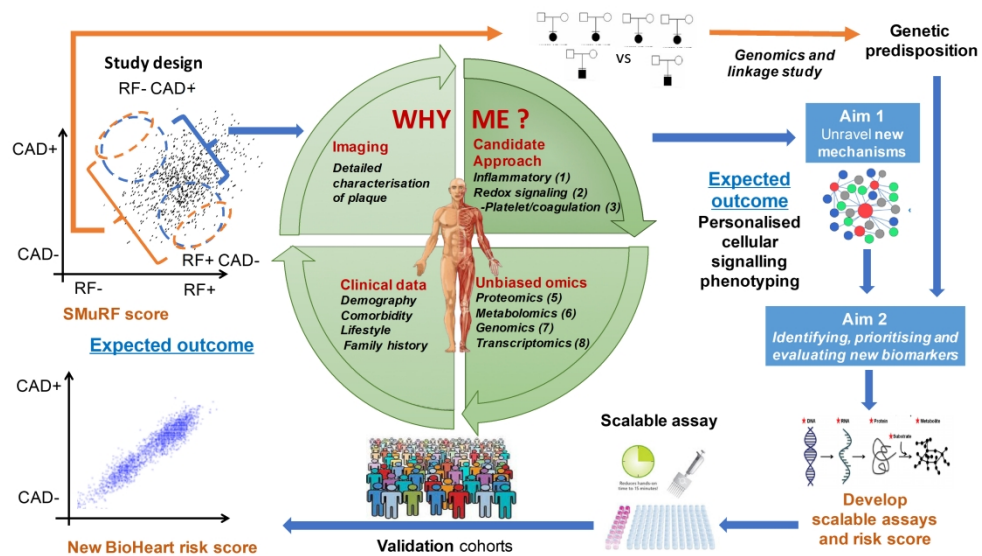


Figure 4: Overview of the Analytic Approach.

358x220mm (300 x 300 DPI)