



**UNIVERSITY OF
KWAZULU-NATAL**

**INYUVESI
YAKWAZULU-NATALI**

**GENETIC AND MICRORNA
POLYMORPHISMS IN YOUNG SOUTH
AFRICAN INDIANS WITH CORONARY
ARTERY DISEASE**

BY

PRITHIKSHA RAMKARAN

B. Sc., B.Med.Sc. (Hons)

*Submitted in fulfillment for the degree of Doctor of Philosophy (Medical Biochemistry), School
of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of
KwaZulu-Natal*

November 2015

DECLARATION

I, Prithiksha Ramkaran declare that:

(i) The research reported in this thesis (except where otherwise indicated): is my original work; has not been submitted for any degree or examination at any other university; does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons; does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

- a) their words have been re-written but the general information attributed to them has been referenced;
- b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced.

(ii) Where I have reproduced a publication of which I am author, co-author or editor, I have indicated in detail which part of the publication was actually written by myself alone and have fully referenced such publications.

(iii) This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the references.

Signed:



10 November 2015

Prithiksha Ramkaran

Date

DEDICATION

To my parents,

Jaychand and Vaniesha Ramkaran

who have always encouraged me to follow my dreams, especially this one...

ACKNOWLEDGEMENTS

One of the joys of looking back is remembering each special individual who has played an important part in supporting me along this journey...

Professor Anil A. Chuturgoon

Thank you doesn't seem sufficient but it is said with appreciation and great respect to you for all your support, care, and encouragement. Thank you for introducing me to the CAD study. Thank you for always having the time to talk not only about science, but life in general. Your precious advice and wise words of wisdom will always be cherished.

Doctor Alisa Phulukdaree

I am extremely lucky to have had you as a mentor, co-supervisor and friend! I appreciate all the time and effort you have put into reading and advising on draft versions of my work. Your hard work and determination is truly inspiring and I will always look up to you as a role model. I only hope to one day make you as proud of me as I am of you.

Doctor Sajidah Khan

Thank you for initiating the CAD study and being my co-supervisor; your hard work and efficiency is second to none and I feel privileged to be a part of this research. Your attention to detail and meticulous documentation of clinical data, made my research possible. Despite your busy schedule, you have always made time for me. Thank you for reading and advising on manuscripts. I really appreciate all that you have done and I look forward to working with you in the future.

A special thanks to all the **patients and controls** who volunteered to be a part of this study.

Doctor Devapregasan Moodley

You are one of the reasons that motivated me to continue with my postgraduate studies. I admire your ability to remain so humble in the face of all your success and achievements. You are truly an inspiration! Thank you for your advice and support on the micro-RNA study, and for making the time to read draft versions of my work.

Discipline of Medical Biochemistry

I appreciate all the helpful suggestions and comments during research meetings. Thank you all for the advice and support. More importantly, thanks for making the lab feel like a home away from home.

Honours 2011/Masters 2012/PhD 2013-2015 Class...

A journey is easier when you travel together, and I am so blessed to have travelled this one with each one of you. We've been there for each other, keeping things in perspective; and at times providing a much needed escape from our studies. You are not just friends to me, you are my extended family. Working with you puts the fun into science, and it has been a pleasure working with such great minds. I would like to wish each of you all the best in future endeavours, and may our friendship continue no matter where we end up.

Scholarships 2011-2015

I would like to thank **The National Research Foundation** and **The College of Health Science** scholarships that provided the financial support necessary for this study

My family and friends

My parents – You have always been so understanding and encouraging towards my studies. Thank you for all the love and support you have given me, and thank you for being such amazing role models. I am eternally grateful for being blessed with having you as parents.

My dearest brother, Pratheer; and sister, Rikara – I am in awe of the amazing young adults you have grown up to be. We have shared good and bad times – and we've been there for each other through it all. Thank you for being so understanding and supportive. May you always have God's blessing.

To the rest of my family and friends – Too many to mention by name, but you each have played a special role in my life. This work would not have been possible without all the love and support you have given me.

PUBLICATIONS

PUBLISHED RESEARCH ARTICLES

1. Phulukdaree A., Khan S., Ramkaran P., Govender R., Moodley D., and Chuturgoon A.A. (2013). The Interleukin-6 -147 G/C Polymorphism Is Associated with Increased Risk of Coronary Artery Disease in Young South African Indian Men. *Metabolic Syndrome and Related Disorders* 11(3), 205-209. (Original article).

(DOI: 10.1089/met.2012.0130)

2. Ramkaran P., Khan S., Phulukdaree A., Moodley D., and Chuturgoon A.A. (2014). miR146a Polymorphism Influences Levels of miR-146a, IRAK-1, and TRAF-6 in Young Patients with Coronary Artery Disease. *Cell Biochemistry and Biophysics* 68(2) 259-268. (Original article).

(DOI: 10.1007/s12013-013-9704-7)

3. Ramkaran, P., Phulukdaree, A., Khan, S., Moodley, D., and Chuturgoon, A.A. (2015). Methylenetetrahydrofolate Reductase C677T polymorphism is associated with increased risk of coronary artery disease in young South African Indians. *Gene* 571(1) 28-32. (Original article).

(DOI: 10.1016/j.gene.2015.06.044)

4. Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. (2015) Sirtuin 1 rs1467568 and rs7895833 in South African Indians with Early-onset Coronary Artery Disease. *Cardiovascular Journal of Africa* (*In press*, October 2015)

(Manuscript number: CVJSA-D-15-00055)

PUBLISHED ABSTRACTS

1. Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. (2014). Methylenetetrahydrofolate Reductase C677T polymorphism is associated with increased risk of coronary artery disease. *South African Heart Journal* 11(4) 200.

2. Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. (2014). MicroRNA-499 Single Nucleotide Polymorphism in Patients with Premature Coronary Artery Disease. *South African Heart Journal* 11(4) 200.

3. Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. (2014). Mitochondrial DNA polymorphisms 5178 C>A and 16189 T>C in young South African Indians with coronary artery disease. South African Heart Journal 11(4) 201.

4. Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. (2014). Sirtuin 1 polymorphisms in young South African Indians with coronary artery disease. South African Heart Journal 11(4) 201.

Manuscript in review:

1. Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. MicroRNA-499 Single Nucleotide Polymorphism in South African Indians with early-onset Coronary Artery Disease. Manuscript submitted to South African Heart Journal (2015).

PRESENTATIONS

1. Ramkaran P., Govender, R., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. Functional analysis of CRP and IL-6 polymorphisms (SNPs) in Indian CVD and Black RA patients. Pfizer-College of Health Sciences Research Symposium, University of KwaZulu-Natal, South Africa, 6th October 2011.
2. Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. miR-146a rs2910164 variant is associated with increased miR-146a expression in young coronary artery disease patients. College of Health Sciences Research Symposium, University of KwaZulu-Natal, South Africa, September 2012.
3. Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. miR-146a Polymorphism Influences Levels of miR-146a, IRAK-1, and TRAF-6 in Young Patients with Coronary Artery Disease. College of Health Sciences Research Symposium, University of KwaZulu-Natal, South Africa, September 2013.
4. Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. MicroRNA-499 Single Nucleotide Polymorphism in South African Indians with early-onset Coronary Artery Disease. College of Health Sciences Research Symposium, University of KwaZulu-Natal, South Africa, September 2014.
5. Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. Methylenetetrahydrofolate Reductase C677T polymorphism is associated with increased risk of coronary artery disease. 15th Annual South African Heart Congress, Durban, South Africa, 16th – 19th October 2014 [Abstract published in South African Heart Journal (2014). Vol. 11(4)] – **Received the Discovery Clinical Excellence Award for the Best Poster Presentation**
6. Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. MicroRNA-499 Single Nucleotide Polymorphism in Patients with Premature Coronary Artery Disease. 15th Annual South African Heart Congress, Durban, South Africa, 16th – 19th October 2014 [Abstract published in South African Heart Journal (2014). Vol. 11(4)].
7. Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. Mitochondrial DNA polymorphisms 5178 C>A and 16189 T>C in young South African Indians with coronary artery disease. 15th Annual South African Heart Congress, Durban, South Africa, 16th – 19th October 2014 [Abstract published in South African Heart Journal (2014). Vol. 11(4)].
8. Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. Sirtuin 1 polymorphisms in young South African Indians with coronary artery disease. 15th Annual South

African Heart Congress, Durban, South Africa, 16th – 19th October 2014 [Abstract published in South African Heart Journal (2014). Vol. 11(4)].

9. Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. microRNA-499 single nucleotide polymorphism in South African Indians with early-onset coronary artery disease. *Accepted Abstract*. 4th International Conference of International Association of Medical and Biomedical Researchers, 9th International Conference of Society of Free Radical Research-Africa (IAMBR-SFRR-Africa), Mauritius 26th – 29th July 2015.

10. Pillay, S., Ramkaran, P., Khan, S., Phulukdaree, A., Moodley, D., and Chuturgoon A. A. MicroRNA-27a rs895819 polymorphism is associated with increased IL-6 levels in coronary artery disease in young South African Indian Patients. College of Health Sciences Research Symposium, University of KwaZulu-Natal, South Africa, September 2015

11. Attending the 11th International Congress on Coronary Artery Disease (29th November – 2nd December, 2015) Florence, Italy.

ABSTRACT

The global burden of cardiovascular disease (CVD) is on the increase with coronary artery disease (CAD) estimated to become the leading cause of mortality worldwide by 2020. The age of onset of this chronic disorder is on the decline, particularly in the South African Indian population. Indians in South Africa (SA) have a higher prevalence of premature CAD compared to other ethnic groups in SA. Coronary artery disease is a lifestyle and genetic disease, and the inheritance of genetic variation from one or both parents plays an important role in the risk of an individual developing CAD. Genetic and epigenetic studies are being explored as potential tools for therapeutic interventions against CVDs. The role of single nucleotide polymorphisms (SNP) in microRNAs (miR, miRNA) and molecules regulating epigenetic pathways remains poorly understood. This study investigated SNPs in candidate genes; methylenetetrahydrofolate reductase (MTHFR), sirtuin (SIRT) 1, miR-499, and miR-146a; in young SA Indians with CAD.

The study population included 106 SA Indian male CAD patients, 100 sex- and age-matched Indian, and 84 sex- and age-matched Black controls. The MTHFR, miR-146a, and miR-499 SNPs were investigated by PCR-RFLP, whilst a TaqMan SNP Genotyping assay assessed the SIRT1 SNPs. MiR-146a expression was measured by qPCR and western blot was used to assess the expression of NF- κ B, IRAK-1, and TRAF-6. Interleukin (IL)-6 levels were analysed using an ELISA. All clinical parameters were obtained from pathology reports.

Methylenetetrahydrofolate reductase is involved in folate metabolism and methylation pathways. The MTHFR rs1801133 has been associated with increased levels of homocysteine, a well known risk factor for CAD. Sirtuin 1, histone deacetylase, has been identified as a candidate molecule affecting the epigenetic mechanisms of CAD. Two common SNPs in the SIRT1 gene, rs7895833 and rs1467568, have been associated with several well-established risk factors for CAD. MicroRNAs (miRNAs) are small noncoding RNA molecules that inhibit messenger RNA (mRNA) translation or promoting mRNA degradation. MiR-499 and miR-146a are inflammatory-associated miRNAs. Two miRNA SNPs, miR-146a rs2910164 and miR-499 rs3746444, have been implicated in chronic inflammatory diseases.

There was a significant association between the MTHFR variant (T) allele and CAD patients compared to Indian controls ($p=0.0353$, OR=2.105 95% CI 1.077–4.114). Indian controls presented with a higher frequency of the T allele compared to Black controls (7% vs. 2% respectively, $p=0.0515$ OR=3.086 95% CI 0.9958–9.564). The variant allele for the two SIRT1 SNPs occurred more frequently in the total Indian group compared to the total Black population (rs1467568: 41% vs. 18.5% respectively, $p<0.0001$, OR=3.190 95% CI 2.058–40943 and rs7895833: 41% vs. 22%

respectively, $p < 0.0001$, OR=2.466 95% CI 1.620–3.755). Indian controls presented with a higher frequency for both SNPs compared to Black controls (rs1467568: 40% vs. 18.5% respectively, $p < 0.0001$, OR=2.996 95% CI 1.850–4.853 and rs7895833: 41% vs. 22% respectively, $p < 0.0001$, OR=2.513 95% CI 1.578–4.004). No difference was seen in the distribution of both SNPs between CAD patients and either control group. The MTHFR and SIRT1 SNPs were not associated with any clinical parameters in CAD patients and controls.

The miR-499 variant (G) allele was found at a higher frequency in the total Indian group (34%) compared to the total Black population (22%) ($p = 0.0070$, OR=1.796 95% CI 1.182–2.730). Indian cases presented with higher frequency of the rs3746444 G allele compared to Indian controls (38% vs. 29%, $p = 0.059$, respectively). No differences in genotypic frequency for rs2910164 was found (GG: 45 vs. 47 %, GC: 46 vs. 41 %, CC: 9 vs. 12 %) in controls and patients respectively (odds ratio=1.025; 95 % confidence interval 0.6782–1.550; $p = 0.9164$).

The lowest levels of NF- κ B and C-reactive protein (hsCRP) were found in patients with the homozygous C allele compared to the heterozygous GC and wildtype variants. Higher levels of miR-499 targets, hsCRP and IL-6, were observed in CAD patients with the variant genotypes compared to those with the wild type genotypes (8.92 ± 1.91 vs. 6.73 ± 0.87 mg/L; $p = 0.299$, 3.02 ± 0.77 vs. 2.18 ± 0.57 pg/mL; $p = 0.381$ respectively). A 6.25-fold increase in miR-146a levels was observed in CAD patients with the CC genotype (relative to controls and patients with the wildtype variant, $p < 0.0001$). These (CC genotype) patients had significantly lower levels of miR-146a targets, IRAK-1 (0.38 ± 0.02 ; $p = 0.0072$) and TRAF-6 (0.44 ± 0.02 ; $p = 0.0146$).

Taken together, this study provides the frequency distribution of the SNPs in four candidate genes for CAD in young South African Indians compared to Indian and Black controls. The frequency of variant alleles of rs1801133, rs3746444, rs7895833 and rs1467568 was greater in the Indian population compared to Black South Africans. Although no difference in frequency was observed for rs2010164, our results suggest a role for miR-146a in toll-like receptor (TLR) signalling via a negative feedback mechanism involving the attenuation of NF- κ B by downregulation of IRAK-1 and TRAF-6. Thus miR-146a can act as a target for therapy towards lowering inflammation in CAD patients.

LIST OF FIGURES

Figure 2.1	A. An overview of the heart and coronary artery. B. Cross-section of the coronary artery showing plaque build-up and a blood clot, leading to dead heart muscle	8
Figure 2.2	The predicted change in the distribution of major causes of mortality worldwide	9
Figure 2.3	CAD mortality in Asian Indians compared with other ethnic groups	12
Figure 2.4	Indian Diabetes Risk Score	16
Figure 2.5	An overview of atherosclerosis in the coronary artery A. Endothelial dysfunction triggered mainly by exposure of the endothelium to oxLDL molecules attracts T-lymphocytes, monocytes, and platelets to the site of injury. B. LDL particles are engulfed by monocytes which thereafter transform into foam cells. Smooth muscle cells migrate from the media, and fatty streaks are formed. C. Accumulation of fatty streaks leads to a fibrous cap. The initial macrophages and monocytes begin to die and form a necrotic core covered by the fibrous cap. D. As the cap increases in size, the artery wall begins to expand until the plaque bulges into the vessel lumen. The fibrous cap eventually thins and ruptures, releasing the contents into the vessel lumen and resulting in thrombus formation. A large enough thrombus can cause luminal occlusion, causing myocardial infarction or stroke	21
Figure 2.6	Biogenesis and processing of miRNAs	28
Figure 2.7	Breakthrough discoveries in miRNA biology. Time line indicating seminal discoveries in miRNA biology with special focus on the cardiovascular field	29
Figure 2.8	A common miR-499 SNP (rs3746444) located within the seed sequence results in a change from A-to-G	31
Figure 2.9	The role of IRAK-1 and TRAF-6 (targets of miR-146a) in the TIR signalling pathway	32
Figure 2.10	Schematic representation of the miR-146a loci and the sequence of mature miR-146a on human chromosome 5	34
Figure 3.1	<i>Hinf</i> I-restricted MTHFR gene fragments. Lane 1: PCR amplicon product, Lane 2 and 3: CT genotype, Lane 4 and 5: CC genotype, Lane 6: TT genotype	55
Figure 5.1	hsCRP and IL-6 levels of CAD patients with the miR-499 rs3746444 wildtype and variant genotypes	85
Figure 6.1	Fold change in miR-146a expression levels in CAD patients relative to controls	99
Figure 6.2	miR-146a expression in CAD patients and controls with different genotypes ***p<0.0001	99
Figure 6.3	IRAK-1 expression in CAD patients (**p=0.0072) and controls	100

Figure 6.4	TRAF-6 expression in CAD patients (*p=0.0146) and controls (#p=0.0247)	100
Figure 6.5	NF-κB expression in CAD patients and controls	101
Figure 6.6	The expression of IRAK-, TRAF-6, and NF-κB in (A) CAD patients compared to (B) Controls.	102
Figure 6.7	The role of miRNA-146a in the inflammatory response through a negative feedback mechanism	103
Appendix 2		
Figure 1	The Polymerase Chain Reaction	116
Appendix 3		
Figure 2	Diagrammatic representations of PCR-RFLP product sizes and corresponding genotypes for A. miR-146a rs2910164, B. MTHFR rs1801133, and C. miR-499 rs3746444. Lane 1 represents the PCR product	121
Appendix 4		
Figure 3	Synthesis of cDNA using the miScript II RT kit	122
Appendix 5		
Figure 4	miR-46a levels in each miR-146a rs2910164 genotype in CAD patients. The CC genotype had the highest miR-146a expression, and therefore the lowest CT value compared to the other genotypes (red: GG, green: GC, blue: CC)	124
Appendix 5		
Figure 5	An example of relative quantification using the comparative threshold cycle (C_t) method	125
Appendix 6		
Figure 6	Protein standardisation by the BCA	127
Appendix 7		
Figure 7	Standard curve derived from the IL-6 standards	130

LIST OF TABLES

Table 2.1	Diagnostic criteria proposed for the clinical diagnosis of MetS	12
Table 2.2	Challenges in identifying genetic determinants of coronary heart disease	23
Table 2.3	Primary features of the miRNA nomenclature system	27
Table 3.1	Frequency of MTHFR C677T genotypes and alleles in SA Indian CAD patients and controls (Indian and Black)	56
Table 3.2	Demographics and clinical parameters for CAD patients, Indian, and Black controls	57
Table 3.3	Characteristics of CAD patients according to MTHFR genotype	58
Table 4.1	SIRT1 rs1467568 and rs7895833 genotype and allele frequencies in CAD patients and controls	70
Table 4.2	Characteristics of CAD patients according to the SIRT1 rs1467568 and SIRT1 rs7895833 genotypes	71
Table 4.3	Characteristics of Indian controls according to the SIRT1 rs1467568 and SIRT1 rs7895833 genotype	72
Table 4.4	Characteristics of Black controls according to the SIRT1 rs1467568 and SIRT1 rs7895833 genotype	73
Table 5.1	miR-499 rs3746444 genotype and allele frequency in CAD patients and controls	84
Table 5.2	Characteristics of CAD patients according to the miR-499 rs3746444 genotype	84
Table 6.1	Demographics and clinical parameters	97
Table 6.2	miR-146a rs2910164 G/C genotype and allele frequency distribution in CAD patients and controls	98
Table 6.3	Mean hsCRP levels (mg/L) between genotypes in CAD patients and controls	101
Appendix 2		
Table 1	miR-146a G/C SNP (rs2010164)	117
Appendix 2		
Table 2	MTHFR C/T SNP (rs1801133)	118
Appendix 2		
Table 3	miR-499 G/C SNP (rs3746444)	119
Appendix 3		
Table 4	Consensus sequence and cleavage site (arrows) for <i>Sac</i> I	120
Appendix 3		
Table 5	Consensus sequence and cleavage site (arrows) for <i>Hinf</i> I	120
Appendix 3		
Table 6	Consensus sequence and cleavage site (arrows) for <i>Bcl</i> I:	120

Appendix 4		
Table 7	Components of reverse transcription reaction	122
Appendix 5		
Table 8	Reaction mix for detection of mature miR-146a	123
Appendix 5		
Table 9	Cycling conditions for the quantitative analysis of mature miR-146a	123
Appendix 7		
Table 10	Preparation of samples of known concentration for the IL-6 ELISA	129

LIST OF ABBREVIATIONS

A	Adenine
AACE	American Association of Clinical Endocrinologists
ABCA1	Adenosine triphosphate (ATP)-binding cassette transporter
ACE-Is	Angiotensin-converting-enzyme inhibitors
ACS	Acute coronary syndrome
ADMA	Asymmetric dimethylarginine
AdoHcy; SAH	S-adenosyl-homocysteine
AdoMet; SAM	S-adenosyl-methionine
AGE	Advanced glycation end products
AMI	Acute myocardial infarction
ANOVA	Analysis of variance
apo A	Apolipoprotein a
apo B-100	Apolipoprotein B-100
BCA	Bicinchoninic Acid
BMI	Body-mass index
bp	Base pair
BP	Blood pressure
BSA	Bovine serum albumin
C	Cytosine
CAD	Coronary artery disease
CD	Cluster of differentiation
cDNA	copy DNA

CHD	Coronary heart disease
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CpG	-Cytosine-phosphate-Guanine-
CR	Calorie restriction
CRP	C-reactive protein
C _t	Comparative threshold cycle
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DDAH	Dimethylarginine dimethylaminohydrolase
DGCR8	DiGeorge syndrome chromosomal (or critical) gene 8
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxynucleoside triphosphates
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
EGIR	European Group for the study of Insulin Resistance
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ESR	Erythrocyte sedimentation rate
FH	Familial hypercholesterolemia
FOXO	Forkhead box O
FXR	Farnesoid X receptor
g	gravitational force

G	Guanine
GWAS	Genome-wide association studies
h	Hour
HAT	Histone acetyl transferase
HbA1c	Glycated haemoglobin
Hcy	Homocysteine
HD	Haemodialysis
HDAC	Histone deacetylase
HDL	High-density lipoprotein
HEP	Human Epigenome Project
HIF	Hypoxia-inducible factor
HRP	Horse radish peroxide
hsCRP	High-sensitivity C-reactive protein
HWE	Hardy-Weinberg equilibrium
IDF	International Diabetes Federation
IDL	Intermediate-density lipoprotein
IDRS	Indian Diabetes Risk Score
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IHEC	International Human Epigenome Consortium
IL	Interleukin
IRAK	IL-1 receptor associated kinase
I κ B	Inhibitor of NF- κ B kinase subunit beta
I κ K	Inhibitor of kappa B

kb	Kilobase
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LNA	Locked nucleic acid
LOX-1	Low-density lipoprotein receptor-1
Lp(a)	Lipoprotein(a)
LXR	Liver X receptor
MCP-1	Monocyte chemoattractant protein
M-CSF	Monocyte-colony stimulating factor
MetS	Metabolic syndrome
MGB	Minor-groove binding
MgCl ₂	Magnesium chloride
MI	Myocardial infarction
min	Minute
miRNA; miR	microRNA
mRNA	messenger RNA
MTHFR	Methylenetetrahydrofolate reductase
MyD88	Myeloid differentiation primary response gene 88
n	Number
NaCl	Sodium chloride
NCD	Noncommunicable diseases
NCEP ATP III	National Cholesterol Education Programme Adult Treatment Panel III
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide

ns	Non-significant
OR	Odds ratio
oxLDL	Oxidised low-density lipoprotein
p53	Tumour suppressor p53
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PGC1- α	Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha
PPAR- γ	Peroxisome proliferator-activated receptor-gamma
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
PTB	Pulmonary tuberculosis
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RAAS	Renin-angiotensin-aldosterone system
RBI	Relative band intensity
RCT	Reverse cholesterol transport
RFLP	Restriction fragment length polymorphism
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rs	Reference SNP
RT	Room temperature
RTC	Reverse cholesterol transport
SA	South Africa

SBP	Systolic blood pressure
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH	Sulfhydryl
SIRT	Sirtuin
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SREBP1c	Sterol regulatory-element binding protein
T	Thymine
T2DM	Type 2 Diabetes mellitus
TAB	TGF- β -activated kinase-binding protein
TAK	TGF- β -activated kinase
<i>Taq</i>	<i>Thermus aquaticus</i>
TAR	Trans-activation response
TARBP	Trans-activation response binding protein
TB	Tuberculosis
TBS	Tris buffered saline
TGF- β	Transforming growth factor-beta
tHcy	Total plasma homocysteine
TIR	Toll-like and IL-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TOLLIP	Toll interacting protein
TRAF	TNF- α receptor associated factor

TRIF	TLR domain-containing adaptor-inducing interferon
Tris-HCl	Tris-Hydrochloric acid
TTBS	Tris-buffered saline containing 0.5% Tween20
U	Uracil
UTR	Untranslated region
UV	Ultra violet
VLDL	Very low-density lipoprotein
WC	Waist circumference
WHO	World Health Organisation

CONTENTS

Declaration	ii
Dedication	iii
Acknowledgements	iv
Publications	vi
Presentations	viii
Abstract	x
List of figures	xii
List of tables	xiv
List of abbreviations	xvi
Chapter 1: Introduction	1
Aims and objectives	3
Research questions	4
Study design	4
Chapter 2: Literature Review	8
2.1 An overview of coronary artery disease	8
2.2 Epidemiology of coronary artery disease	9
2.3 Risk factors for coronary artery disease	9
2.3.1 Non-modifiable risk factors	10
(a) Age	10
(b) Gender	10
(c) Genetic/Hereditary	10
• Familial Hypercholesterolemia	11
(d) Ethnicity	11

2.3.2 Modifiable risk factors	13
(a) Diabetes Mellitus and Metabolic Syndrome	13
(b) Tobacco	16
(c) Hypertension	17
(d) Dyslipidemia	18
(e) Diet, obesity, and physical inactivity	18
2.3.3 “Novel” risk factors	19
(a) Excess homocysteine	19
(b) C-reactive protein	20
(c) Lipoprotein(a)	20
2.4 Pathogenesis of coronary artery disease: Atherosclerosis – Roles of inflammation, endothelial perturbation and lipids	21
2.5 Genetic variation in coronary artery disease	23
2.5.1 Association studies: Identifying genes related to coronary artery disease – The candidate gene approach	24
2.6 Epigenetics and coronary artery disease	25
2.6.1 DNA modification	25
2.6.2 Histone modification	26
2.6.3 MicroRNAs	26
2.6.3.1 MicroRNA biogenesis and processing	27
2.6.3.2 MicroRNAs in coronary artery disease	29
2.6.3.3 Single nucleotide polymorphisms in microRNA genes	30
(a) miR-499 and rs3746444	30
(b) miR-146a and rs2910164	32

Chapter 3: Methylenetetrahydrofolate Reductase C677T polymorphism is associated with increased risk of coronary artery disease in young South African Indians

Ramkaran, P., Phulukdaree, A., Khan, S., Moodley, D., and Chuturgoon, A.A. (2015) Methylenetetrahydrofolate Reductase C677T polymorphism is associated with increased risk of coronary artery disease in young South African Indians. *Gene* 571(1) 28-32. (Original article)

(DOI: 10.1016/j.gene.2015.06.044) 49

Chapter 4: Sirtuin 1 rs1467568 and rs7895833 in South African Indians with Early-onset Coronary Artery Disease

Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. (2015) Sirtuin 1 rs1467568 and rs7895833 in South African Indians with Early-onset Coronary Artery Disease. *CardioVascular Journal of Africa (In press, October 2015)*

(Manuscript number: CVJSA-D-15-00055) 64

Chapter 5: MicroRNA-499 Single Nucleotide Polymorphism in South African Indians with early-onset Coronary Artery Disease

Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. MicroRNA-499 Single Nucleotide Polymorphism in South African Indians with early-onset Coronary Artery Disease.

Manuscript in review: *South African Heart Journal* (2015) 78

Chapter 6: miR-146a Polymorphism Influences Levels of miR-146a, IRAK-1, and TRAF-6 in Young Patients with Coronary Artery Disease

Ramkaran P., Khan S., Phulukdaree A., Moodley D., and Chuturgoon A.A. (2014) miR146a Polymorphism Influences Levels of miR-146a, IRAK-1, and TRAF-6 in Young Patients with Coronary Artery Disease. *Cell Biochemistry and Biophysics* 68(2) 259-268. (Original article)

(DOI: 10.1007/s12013-013-9704-7) 90

Chapter 7: Conclusion 108

Appendix 1: Ethics Statement 115

Appendix 2: Polymerase Chain Reaction 116

Optimisation of Polymerase Chain Reaction 117

Appendix 3: Restriction Fragment Length Polymorphism 120

Appendix 4: Synthesis of cDNA	122
Appendix 5: Quantitative Polymerase Chain Reaction	123
Analysis of Quantitative Polymerase Chain Reaction Results	124
Appendix 6: Protein Isolation	126
The Bicinchoninic Acid Assay	126
Preparation of samples for Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis	127
Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis and Western Blot	127
Analysis of Western Blot	128
Appendix 7: Interleukin-6 Enzyme-Linked Immunosorbent Assay	129
Appendix 8: TaqMan[®] SNP Genotyping Assay	131
Appendix 9: Acceptance letter for Manuscript - Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. (2015) Sirtuin 1 rs1467568 and rs7895833 in South African Indians with Early-onset Coronary Artery Disease. CardioVascular Journal of Africa (<i>In press</i>, October 2015) (Manuscript number: CVJSA-D-15-00055)	132

CHAPTER 1

INTRODUCTION

Thirty-eight million people worldwide die annually from non-communicable diseases (NCDs), mainly from cardiovascular diseases (CVDs). According to the World Health Organization (WHO), CVD kills approximately 17.5 million people each year, increasing to 25 million deaths annually in the next few years (Mendis, Armstrong et al. 2014). Coronary artery disease (CAD), which is the most important contributor to CVD, is expected to become the number one cause of mortality worldwide by 2020 (Levenson, Skerrett et al. 2002). Cardiovascular disease has been the leading cause of death in developed countries for most of the last century, however, the incidence of CAD in developing countries such as South Africa (SA) is on the rise (Okraïneç, Banerjee et al. 2004).

Evidence from mortality studies indicate that the global burden of CVDs is not shared equally across ethnic groups (Murray and Lopez 1997; Yusuf, Reddy et al. 2001). Indian populations worldwide have the highest prevalence of early-onset CAD compared to other ethnic groups (Rajeshwari, Nicklas et al. 2005; Khan 2013). Early-onset or premature CAD is defined as cardiac events occurring before the age of 55 in men and 65 in women, and before the age of 40 in its severe form (Rissam, Kishore et al. 2001). People of Indian ancestry in particular develop heart disease when they are younger than 40, a phenomenon unheard of in other populations (Sastry, Golla et al. 2011).

The pattern with regard to early-onset CAD in the Indian population has been similar in various parts of the world for decades. In India, 25–40% of patients suffering from acute myocardial infarction (AMI) were below the age of 45 (Girija 1997). In Great Britain, AMI occurred in Indians younger than 40 years old — and the incidence was 10 times higher than the local Caucasian population (Rajadurai, Arokiasamy et al. 1992). Studies in Singapore reported that mortality due to CAD below 30 years of age occurs 10 times more in the Indian population compared to the local Chinese (Hughes, Lun et al. 1990). In the Middle East, 80% of patients who experienced AMI were Indians below the age of 40, yet Indians account for approximately 10% of the population in this region. Angiographically, Indians have a 15 times higher rate of CAD than Chinese and 10 times higher rate than Malays below 40 years of age (Rissam, Kishore et al. 2001).

In 1966, Wainwright reported a high incidence of CAD in SA Indians (Wainwright 1966). In 2006, reports showed that the highest death rates for CAD in SA occur in Indians, followed by mixed race, White, and Black (Norman, Schneider et al. 2006). This suggests that regardless of the number of generations of Indian immigrants who have lived in SA, there remains a differing pattern of CAD mortality compared to the natives of the country, despite them sharing the same environment (Lal 2004).

Coronary artery disease is a multifactorial disease that depends on the interaction between environmental risk factors and several predisposing genes (Andreassi, Botto et al. 2003). On a genetic basis, single nucleotide polymorphisms (SNP) in key genes may play a vital role in an individual's susceptibility and response to the disease. The role of SNPs in protein coding genes has been well investigated, but remains poorly understood in microRNAs (miRNAs; miRs) and molecules regulating epigenetic pathways.

Epigenetics refers to mechanisms that regulate gene expression that do not involve alteration in DNA sequence. Three areas of epigenetics exist: DNA modification, histone modification, and RNA-based mechanisms (Pons, de Vries et al. 2009; Webster, Yan et al. 2013). The epigenetic pathways are complex and interconnected, and involve highly regulated molecules. Epigenetic research is relatively new but rapidly growing, and may provide novel insight into the complex mechanisms underlying CAD.

Hyperhomocysteinaemia is a well-established risk factor for CAD. Methylene tetrahydrofolate reductase (MTHFR) reduces 5',10'-methylene tetrahydrofolate to 5'-methyl tetrahydrofolate, and is involved in remethylation of homocysteine to methionine, two important reactions involved in folate metabolism and methylation pathways. The common MTHFR C677T single nucleotide polymorphism (SNP) (rs1801133) has been associated with raised levels of homocysteine, a well known risk factor for CAD.

Sirtuin (SIRT) 1, a class III histone deacetylase, has been identified as a candidate molecule affecting the epigenetic mechanisms of CVD. Previous studies have shown that some SIRT 1 SNPs are associated with inflammation, body mass index (BMI), type 2 diabetes (T2DM), blood pressure, and dyslipidemia, all of which are well-established risk factors for CAD (Finkel, Deng et al. 2009; Horio, Hayashi et al. 2011; Cui, Wang et al. 2012; Houtkooper, Pirinen et al. 2012).

MicroRNAs are a class of small noncoding RNA molecules that negatively regulate gene expression of target genes by inhibiting messenger RNA (mRNA) translation or promoting mRNA degradation (Corsten, Dennert et al. 2010; Wang, Jiao et al. 2011). A single miR is able to regulate multiple target genes whereas one target gene may be regulated by various miRs (Li, Gao et al. 2011). MicroRNAs regulate most biological processes, therefore aberrant miR expression is likely to be implicated in several pathological conditions (Zhou, Rao et al. 2010; Yang, Chen et al. 2012). Genetic variation such as SNPs in miRs may affect their processing, expression and interaction with target genes (Li, Gao et al. 2011). Altered miR expression has been well investigated in various cancer types (Qiu, Hu et al. 2012; Li, Sheng et al. 2013), but miRs in cardiovascular disease is still an emerging field of research with very few studies focusing on coronary artery disease (Ono, Kuwabara et al. 2011). MicroRNA-499 and miR-146a are two of several miRs implicated in chronic inflammatory diseases such as CAD.

Several cytokines, including interleukin (IL)-17 receptor β (IL-17R β), IL-23a, IL-2 β , IL-6, IL-2, IL-18R, and IL-21 are well known targets of miR-499 (Yang, Chen et al. 2012; Hashemi, Eskandari-Nasab et al. 2013). MicroRNA-499 has been implicated in inflammatory diseases such as rheumatoid arthritis (RA), and pulmonary tuberculosis (PTB) (Li, Wang et al. 2011; Yang, Chen et al. 2012). A common miR-499 A/G SNP (rs3746444) located within the stem region opposite the mature miR-499 sequence, results in a change from an A:U pair to a G:U mismatch in the stem structure of miR-499 (Wang, Tian et al. 2013). The variant G allele has a less stable secondary structure than the wildtype allele and directly affects the binding to target mRNAs and the miR maturation process, therefore altering protein expression and potentially contributing to disease susceptibility.

miR-146a is involved in regulating the innate immune, and inflammatory response pathways (Zilahi, Tarr et al. 2011). A common G/C SNP (rs2910164) located within the crucial seed sequence of miR-146a (Jazdzewski, Liyanarachchi et al. 2009) causes a change from a G:U pair to a mismatched C:U in the stem region and was shown to affect the expression of mature miR-146a (Xu, Zhu et al. 2008; Hung, Chang et al. 2012). Whether genetic variants of miR-146a confer differences in expression of miR-146a in CAD is unclear (Chatzikiyriakidou, Voulgari et al. 2010; Yue, Wang et al. 2011; Hung, Chang et al. 2012). If such differences do occur, whether they are enough to produce significantly different regulatory effects on major mRNA targets remains unknown.

The role of SNPs in inflammatory miRs such as miR-499 and miR-146a, and epigenetic regulators such as MTHFR and SIRT1 has not been described in SA Indians with CAD. Research identifying the role of genetic factors in determining susceptibility to CAD in the SA Indian population is needed. These genetic studies may contribute to a greater understanding of the genomic and epigenetic drivers of heterogeneity in individual responses to CAD, paving the way for personalised medical treatment (Mocumbi 2015). Discovery of genetic susceptibility loci may provide a clinically useful genetic risk-prediction tool that will potentially allow identification of those at higher risk for CAD and early administration of therapeutics.

Aims and objectives

The overall aim of this study was to assess selected genetic and miRNA polymorphisms that may play a role in the susceptibility of early-onset CAD in South African Indians.

The specific objectives of this study were designed to assess:

- (i) MTHFR rs1801133 (Chapter 3)
- (ii) SIRT1 rs1467568 and rs7895833 (Chapter 4)
- (iii) miR-499 rs3746444 (Chapter 5)

(iv) miR-146a rs2910164 (Chapter 6)

Research questions

- What are the frequencies of these SNPs in young SA Indians with CAD compared to Indian and Black controls?
- Do these SNPs influence any clinical parameters?
- Do the miR SNPs affect target gene expression?
- Are these SNPs associated with CAD?
- Do these molecules show any potential as biomarkers for therapeutic intervention against CAD?

Study design

A case-control study was designed to achieve the research objectives. Cases with angiographically documented CAD, that met the inclusion requirements, were enrolled into the study (Inkosi Albert Luthuli Central Hospital, Durban, South Africa). Control subjects were randomly selected, and those that met the inclusion criteria were recruited into this study. To be included into this study, cases needed to match the following criteria: Indian ancestry and unrelated, adults aged below 45 years and stable CAD confirmed on angiography. Exclusion criteria for patients were an acute coronary syndrome/ revascularisation procedure in the preceding three months, chronic renal or liver disease, malignancy and known active inflammatory or infectious disease. Indian and Black male controls that did not have heart disease were recruited. Inclusion criteria for controls were Indian/African ancestry, unrelated to one another, adult males below the age of 45 years and no known or suspected atherosclerotic vascular disease (Khan 2013).

The present study was approved by the Biomedical Research Ethics Committee of KwaZulu-Natal (Ethics number: BE067/14), and conducted in accordance with the ethical principles described by the Declaration of Helsinki. After being well-informed about the study, all participants gave written informed consent.

A total of 106* young SA Indian male CAD patients (mean age 37.5, range 24–45 years), 100* Indian male controls (mean age 37.5, range 28–45 years), and 84* Black male controls (mean age 36.4, range 25–45) were enrolled in this study. Recruitment numbers were adequate for a 95% level of confidence and an 80% power to detect statistical differences.

**Numbers may differ in subsequent chapters according to success of PCR amplification.*

References

- Andreassi, M., N. Botto, et al. (2003). "Methylenetetrahydrofolate reductase gene C677T polymorphism, homocysteine, vitamin B12, and DNA damage in coronary artery disease." Human Genetics 112(2): 171-177.
- Chatzikiyiakidou, A., P. V. Voulgari, et al. (2010). "A polymorphism in the 3'-UTR of interleukin-1 receptor-associated kinase (IRAK1), a target gene of miR-146a, is associated with rheumatoid arthritis susceptibility." Joint Bone Spine 77(5): 411-413.
- Corsten, M. F., R. Dennert, et al. (2010). "Circulating MicroRNA-208b and MicroRNA-499 Reflect Myocardial Damage in Cardiovascular Disease." Circulation: Cardiovascular Genetics 3(6): 499-506.
- Cui, Y., H. Wang, et al. (2012). "Genetic analysis of the SIRT1 gene promoter in myocardial infarction." Biochemical and Biophysical Research Communications 426(2): 232-236.
- Finkel, T., C.-X. Deng, et al. (2009). "Recent progress in the biology and physiology of sirtuins." Nature 460(7255): 587-591.
- Girija, G. (1997). Cardiovascular Disease Prevention - American Heart Association Heart and Stroke Statistical Update. 26: 78-83.
- Hashemi, M., E. Eskandari-Nasab, et al. (2013). "Association of pre-miRNA-146a rs2910164 and premiRNA-499 rs3746444 polymorphisms and susceptibility to rheumatoid arthritis." Molecular Medicine Reports 7: 287-291.
- Horio, Y., T. Hayashi, et al. (2011). "Cellular and molecular effects of sirtuins in health and disease." Clinical Science 121(5): 191-203.
- Houtkooper, R. H., E. Pirinen, et al. (2012). "Sirtuins as regulators of metabolism and healthspan." Nat Rev Mol Cell Biol 13(4): 225-238.
- Hughes, K., K. C. Lun, et al. (1990). "Cardiovascular diseases in Chinese, Malays, and Indians in Singapore. I. Differences in mortality." Journal of Epidemiology and Community Health 44(1): 24-28.
- Hung, P. S., K. W. Chang, et al. (2012). "Association between the rs2910164 polymorphism in pre-mir-146a and oral carcinoma progression." Oral Oncology 48: 404-408.
- Jazdzewski, K., S. Liyanarachchi, et al. (2009). "Polymorphic mature microRNAs from passenger strand of pre-miR-146a contribute to thyroid cancer." Proceedings of the National Academy of Sciences 106(5): 1502-1505.
- Khan, S. (2013). Telomere Dynamics in Atherosclerosis. Doctor of Philosophy, University of KwaZulu-Natal.
- Lal, S. (2004). Genetic epidemiology of coronary artery disease. Master of Science, National University of Singapore.

- Levenson, J. W., P. J. Skerrett, et al. (2002). "Reducing the Global Burden of Cardiovascular Disease: The Role of Risk Factors." Preventive Cardiology 5(4): 188-199.
- Li, D., T. Wang, et al. (2011). "Genetic study of two single nucleotide polymorphisms within corresponding microRNAs and susceptibility to tuberculosis in a Chinese Tibetan and Han population." Human Immunology 72(7): 598-602.
- Li, L., Y. Sheng, et al. (2013). "The Association between Two MicroRNA Variants (miR-499, miR-149) and Gastrointestinal Cancer Risk: A Meta-Analysis." PLoS ONE 8(11): e81967.
- Li, L. J., L. B. Gao, et al. (2011). "Association between SNPs in pre-miRNA and risk of chronic obstructive pulmonary disease." Clinical Biochemistry 44: 813-816.
- Mendis, S., T. Armstrong, et al. (2014). Global status report on noncommunicable diseases. W. H. Organization. Geneva, Switzerland.
- Mocumbi, A. O. (2015). "Rheumatic heart disease in Africa: is there a role for genetic studies?" Cardiovascular Journal of Africa 26(2): 21-26.
- Murray, C. J. and A. D. Lopez (1997). "Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study." The Lancet 349: 1498-1504.
- Norman, R. B. D., M. Schneider, et al. (2006). Revised Burden of Disease Estimates for the comparative risk factor assessment, South Africa 2000. Cape Town: Medical Research Council 2006.
- Okrainec, K., D. K. Banerjee, et al. (2004). "Coronary artery disease in the developing world." American Heart Journal 148(1): 7-15.
- Ono, K., Y. Kuwabara, et al. (2011). "MicroRNAs and cardiovascular diseases." The FEBS Journal 278: 1619-1633.
- Pons, D., F. R. de Vries, et al. (2009). "Epigenetic histone acetylation modifiers in vascular remodelling: new targets for therapy in cardiovascular disease." European Heart Journal 30: 266-277.
- Qiu, M.-T., J.-W. Hu, et al. (2012). "Hsa-miR-499 rs3746444 Polymorphism Contributes to Cancer Risk: A Meta-Analysis of 12 Studies." PLoS ONE 7(12): e50887.
- Rajadurai, J., J. Arokiasamy, et al. (1992). "Coronary artery disease in Asians." Australian and New Zealand Journal of Medicine 22(4): 345-348.
- Rajeshwari, R., T. A. Nicklas, et al. (2005). "Cardiovascular diseases—a major health risk in Asian Indians." Nutrition research (New York, N.Y.) 25(6): 515-533.
- Rissam, H. S., S. Kishore, et al. (2001). "Coronary Artery Disease in Young Indians - The Missing Link." Journal, Indian Academy of Clinical Medicine 2(3).
- Sastry, B. K. S., J. Golla, et al. (2011). "Genetic interactions between MTHFR (C677T), methionine synthase (A2756G, C2758G) variants with vitamin B12 and folic acid determine susceptibility to premature coronary artery disease in Indian population." Journal of Cardiovascular Disease Research 2(3): 156-163.

- Wainwright, J. (1966). "Cardiovascular disease in the Asiatic (Indian) population of Durban." South African medical journal 43(6): 136-138.
- Wang, J. X., J. Q. Jiao, et al. (2011). "miR-499 regulates mitochondrial dynamics by targeting calcineurin and dynamin-related protein-1." Nature Medicine 17: 71-79.
- Wang, N., Z. Q. Tian, et al. (2013). "An A/G polymorphism rs3746444 in miR-499 is associated with increased cancer risk: a meta-analysis." Genetics and Molecular Research 12(3): 3955-3964.
- Webster, A. L. H., M. S. C. Yan, et al. (2013). "Epigenetics and Cardiovascular Disease." Canadian Journal of Cardiology 29: 46-57.
- Xu, T., Y. Zhu, et al. (2008). "A functional polymorphism in the miR-146a gene is associated with the risk for hepatocellular carcinoma." Carcinogenesis 29(11): 2126-2131.
- Yang, B., J. Chen, et al. (2012). "Association of polymorphisms in pre-miRNA with inflammatory biomarkers in rheumatoid arthritis in the Chinese Han population." Human Immunology 73: 101-106.
- Yue, C., M. Wang, et al. (2011). "Polymorphism of the pre-miR-146a is associated with risk of cervical cancer in a Chinese population." Gyneologic Oncology 122: 33-37.
- Yusuf, S., S. Reddy, et al. (2001). "Global Burden of Cardiovascular Diseases: Part I: General Considerations, the Epidemiologic Transition, Risk Factors, and Impact of Urbanization." Circulation 104: 2746-2753.
- Zhou, B., L. Rao, et al. (2010). "Common genetic polymorphisms in pre-microRNAs were associated with increased risk of dilated cardiomyopathy." Clinica Chimica Acta 411(17-18): 1287-1290.
- Zilahi, E., T. Tarr, et al. (2011). "Increased microRNA-146a/b, TRAF6 gene and decreased IRAK1 gene expressions in the peripheral mononuclear cells of patients with Sjögren's syndrome." Immunology Letters 141: 165-168.

CHAPTER 2

LITERATURE REVIEW

2.1 An overview of coronary artery disease

The coronary artery system is responsible for delivering oxygen- and nutrient-rich blood to the heart. Coronary artery disease (CAD), sometimes referred to as coronary heart disease (CHD), is a manifestation of atherosclerosis, which results in the coronary bed system failing to provide adequate blood supply to the heart due to the accumulation of atheromatous plaques within the walls of the coronary arteries (Figure 2.1) (Hansson 2005; Dzimir, Wang et al. 2012). Atherosclerosis is a multifactorial disease characterised by chronic inflammation from initiation to progression, whereby immune and metabolic factors interact to propagate and activate arterial lesions (Hansson 2005). This process is under genetic and epigenetic regulation (Sayols-Baixeras, Lluís-Ganella et al. 2014).

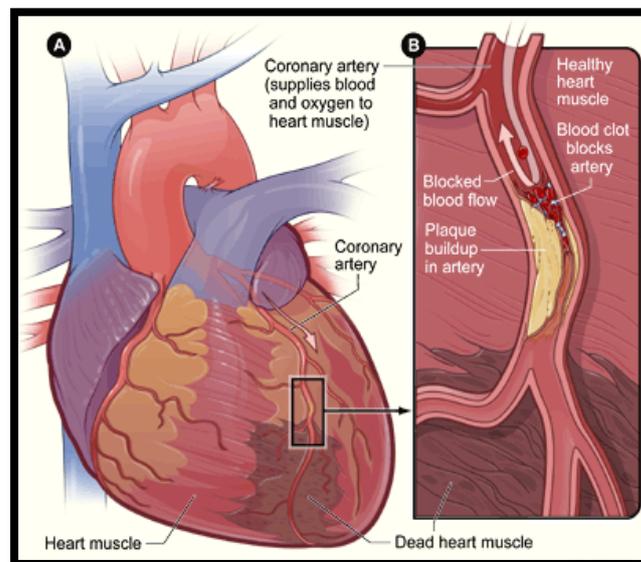


Figure 2.1 A. An overview of the heart and coronary artery. B. Cross-section of the coronary artery showing plaque build-up and a blood clot, leading to dead heart muscle (NHLBI 2013) (http://www.nhlbi.nih.gov/sites/www.nhlbi.nih.gov/files/images_259)

According to the American College of Cardiology and American Heart Association guidelines for coronary angiography, CAD is defined as the presence of $\geq 50\%$ diameter stenosis in one or more major coronary artery and is classified as single, double, or triple-vessel disease; depending on the number of major coronary arteries affected (Scanlon P.J., Faxon D.P. et al. 1999).

2.2 Epidemiology of coronary artery disease

The World Health Organisation (WHO) recently reported that noncommunicable diseases (NCDs) are the main cause of mortality worldwide, responsible for thirty-eight million annual deaths (World Health Organization, 2014). More than 17 million NCD deaths were due to cardiovascular disease (CVD) (46.2% of NCD deaths), and is projected to rise to 25 million in 2020 (Figure 2.2), surpassing infectious disease as the leading cause of mortality worldwide (Levenson, Skerrett et al. 2002; Fuster V and BB 2010). Coronary artery disease, the single most important contributor to CVD, is expected to become the number one cause of death worldwide by 2020 (Murray and Lopez 1997; Okraïneç, Banerjee et al. 2004; Dahlöf 2010). By this time, developing countries are expected to contribute 85% towards the global burden of CAD (Murray and Lopez 1997).

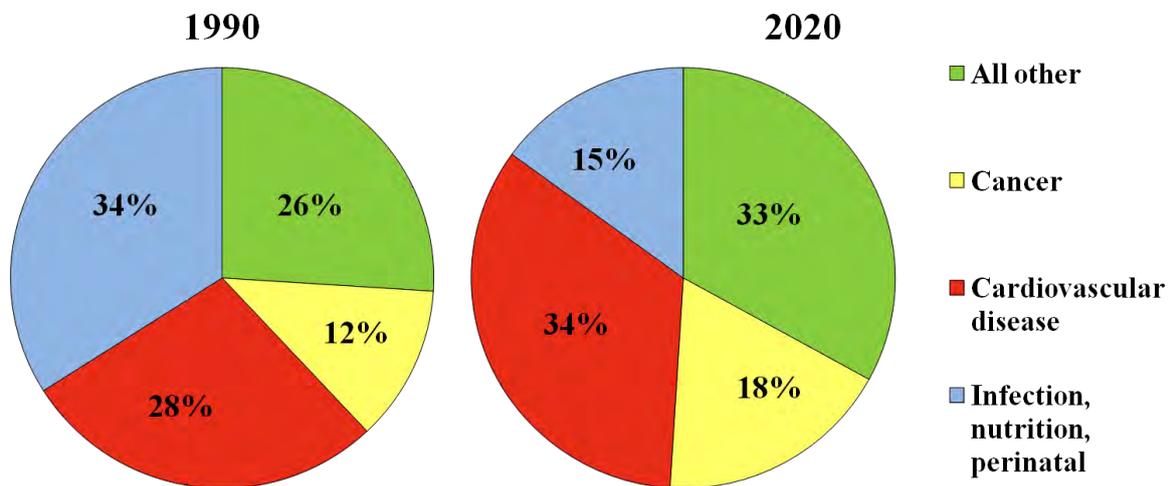


Figure 2.2 The predicted change in the distribution of major causes of mortality worldwide (Levenson, Skerrett et al. 2002)

2.3 Risk factors for coronary artery disease

Extensive research has identified several factors that increase the risk of developing CAD. Some risk factors cannot be changed (non-modifiable risk factors), while others can be treated or changed (modifiable risk factors).

2.3.1 Non-modifiable risk factors

(a) Age

Atherosclerosis is a cumulative process, beginning at a fairly young age. As the disease progresses with age, the risk of CAD increases (Jousilahti, Vartiainen et al. 1999). However, this pattern is changing as younger individuals are now presenting with early-onset CAD (Sharma M 2005; Khan 2013). Early-onset or premature CAD is defined as cardiac events occurring before the age of 55 in men and 65 in women, and before the age of 40 in its severe form (Rissam, Kishore et al. 2001). People of Indian ancestry in particular develop heart disease when they are younger than 40, a phenomenon unheard of in other populations (Enas, V et al. 2007; Aggarwal, Aggarwal et al. 2012).

(b) Gender

Among middle-aged people, CAD is 2-5 times more common in men than in women, with a varying sex ratio between populations (Jousilahti, Vartiainen et al. 1999). Oestrogens provide a protective effect, but this is reduced once a woman reaches menopause, and her risk for CAD increases (Achari and Thakur 2004). The decrease in oestrogen production after menopause alters the female lipid profile, favouring a more atherogenic form by decreasing high-density lipoprotein (HDL) cholesterol and increasing low-density lipoprotein (LDL) cholesterol, total cholesterol, triglycerides (TG) , and lipoprotein(a) (Lp(a)) levels. In addition to having an effect on lipids, oestrogen may have cardioprotective effects via glucose metabolism as well as a direct effect on endothelial cell function. (Jousilahti, Vartiainen et al. 1999). The risk of CAD in women increases with the use of tobacco and oral contraceptives (Klein and Nathan 2003).

A recent study involving more than 3000 British men explored the role of the Y chromosome in CAD (Charchar, Bloomer et al. 2012; Mearns 2012). They identified nine haplotypes, one of which (haplotype 1) had a 50% higher risk of CAD than the other Y chromosome lineages. Importantly, this association was independent of traditional cardiovascular risk factors such as body-mass index (BMI), blood pressure, lipids, diabetes, smoking, alcohol consumption, or circulating levels of C-reactive protein (CRP). Men who inherited haplotype 1 from their male ancestors had a downregulation of adaptive immunity and an upregulated inflammatory response compared to those who carried the other Y chromosome lineages (Charchar, Bloomer et al. 2012).

(c) Genetic/Hereditary

A positive family history is a well known independent risk factor for CAD (Schächinger, Britten et al. 1999; Sadeghi, Adnani et al. 2013). Coronary artery disease is a genetic disease, and the inheritance of genetic variation from one or both parents plays an important role in the risk of an individual

developing CAD. The associated risk further increases when first-degree relatives are affected at a younger age, with an odds ratio (OR) of 1.3 in individuals with relatives affected <55 years, to ORs of 10 and greater in individuals with affected relatives <45 years (Sesso, Lee et al. 2001; Nasir, Michos et al. 2004).

- **Familial Hypercholesterolaemia**

Familial hypercholesterolaemia (FH) is associated with increased risk of CAD due to the genetically determined increased LDL cholesterol in these individuals (Narverud, Retterstol et al. 2014). The disorder is caused by the absence of or defective LDL receptors (LDLR) which occurs as a result of polymorphisms in the LDLR gene. The heterozygous form of this disorder occurs in 1 in 500 people who present with total cholesterol concentration in excess of 300 mg/dL. Approximately 5% of all patients who present with acute MI (AMI) before 60 years of age have heterozygous FH. The homozygous form of FH occurs in about 1 in 1 million individuals and presents with cholesterol levels ranging from 600-1000 mg/dL. These patients usually develop CAD before the age of 20 (Crowther 2005).

(d) Ethnicity

Ethnic and regional variations exist as risk factors for developing CAD (Figure 2.4) (Enas A Enas, Annamalai Senthilkumar et al. 2005; Dzimiri, Wang et al. 2012). People originating from the Indian subcontinent show unusually high incidence and mortality due to CAD. Asian Indians have the highest susceptibility in developing CAD, 3-4 times more than Caucasians, 6 times more than Chinese, and 20 times more than Japanese (Rajeshwari, Nicklas et al. 2005).

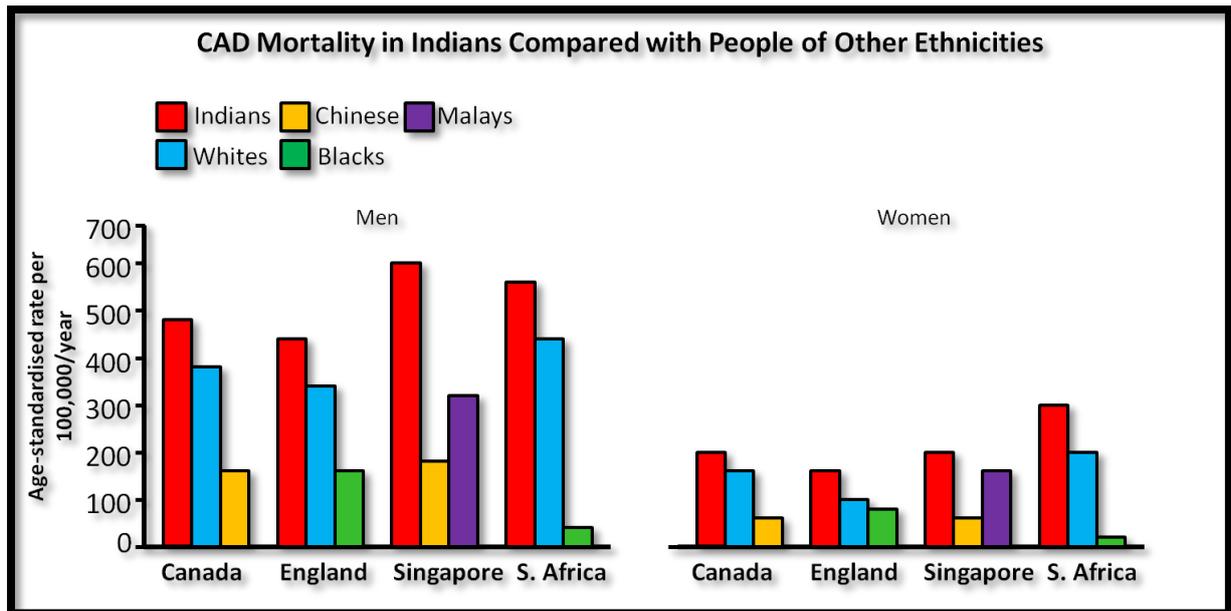


Figure 2.3 CAD mortality in Asian Indians compared with other ethnic groups (Enas A Enas, Annamalai Senthilkumar et al. 2005)

Most South Africans of Indian ethnicity are descendants of indentured labourers who were brought to KwaZulu-Natal from India between 1860 and 1911 (Seedat 2005). Indians in India and South Africa (SA) are predisposed to the early onset of CAD, one to two decades earlier than other population groups, indicating a genetic link (Ranjith, Pegoraro et al. 2005; Sharma M 2005). In 1966, Wainwright reported a high incidence of CAD in South African Indians (Wainwright 1966). In 2006, reports still showed that the highest death rates for CAD in SA occur in Indians, followed by mixed race, White, and Black (Norman, Schneider et al. 2006). This suggests that regardless of the number of generations of Indian immigrants who have lived in SA, there remains a differing pattern of CAD mortality compared to the natives of the country, despite them sharing the same environment. (Lal 2004).

Indians develop CAD at a much younger age (younger than 40 years of age) compared to other ethnicities. Indian populations throughout the world have the highest mortality rate due to CAD compared with people of other ethnicities (Figure 1.4) (Enas A Enas, Annamalai Senthilkumar et al. 2005). For decades, the pattern with regard to early-onset CAD in the Indian population has remained similar in various parts of the world.

In India, 25-40% of patients suffering an AMI were below the age of 45 (Girija 1997). In Great Britain, AMI occurred in Indians younger than 40 years old – and the incidence was 10 times higher than the local Caucasian population (Rajadurai, Arokiasamy et al. 1992). Studies in Singapore reported mortality due to CAD below 30 years of age occurs 10 times more in the Indian population

compared to the local Chinese (Hughes, Lun et al. 1990). In the Middle East, 80% of patients who experienced AMI were Indians below the age of 40, yet Indians account for approximately 10% of the population in this region (Rissam, Banzal et al. ; Rissam, Kishore et al. 2001).

Angiographically, Indians have a 15 times higher rate of CAD than Chinese and 10 times higher rate than Malays below 40 years of age (Rissam, Kishore et al. 2001). The disease is also much more severe in Indian cases. Compared to other ethnicities, young Indians often present with triple vessel disease with poor prognosis. Compared to Caucasians, Indians have a 3 times higher rate of re-infarction and 2 times higher rate of mortality (Danaraj, Acker et al. 1959; Sharma, Kaul et al. 1990; Wilkinson, Sayer et al. 1996; Wild and McKeigue 1997).

2.3.2 Modifiable risk factors

(a) Diabetes mellitus and Metabolic syndrome

Type 2 Diabetes mellitus (T2DM) is a multifactorial disease characterised by hyperglycaemia due to impaired insulin secretion, and may be acquired or inherited (Das and Elbein 2006). There is a strong genetic component in T2DM, where the risk of developing T2DM is substantially increased if the individual has first degree family with T2DM (Lin and Sun 2010; Olokoba, Obateru et al. 2012). An individual is further at risk for developing CAD if they are diabetic. Hyperglycaemia can lead to modification of macromolecules, by forming advanced glycation end products (AGE). By binding to surface receptors, AGE activate inflammatory pathways and upregulate the production of proinflammatory cytokines. The diabetic state also promotes oxidative stress, which perpetuates the inflammatory response (Libby, Ridker et al. 2002).

Metabolic syndrome (MetS) is a complex disorder characterised by several integrated physiological, biochemical, clinical, and metabolic factors that directly increase risk for T2DM and CVD (Weng 2005, Palmieri 2011). The exact mechanisms underlying the pathways of MetS are under investigation. The most important factors include genetics, epigenetics, aging, diet, obesity, and sedentary lifestyle (Kaur 2014). In addition to dyslipidaemia, hypertension, insulin resistance, and hyperglycaemia, the syndrome presents with a prothrombotic and proinflammatory state (Grundy 2008).

Various definitions of MetS have been proposed by different organisations over the past decade. The most common criteria for MetS currently used are from the WHO, the European Group for the study of Insulin Resistance (EGIR), the National Cholesterol Education Programme Adult Treatment Panel

III (NCEP ATP III), American Association of Clinical Endocrinologists (AACE), and the International Diabetes Federation (IDF) (Alberti, Eckel et al. 2009; Kaur 2014) (Table 2.1).

Table 2.1 Diagnostic criteria proposed for the clinical diagnosis of MetS (Kaur 2014)

Clinical measures	WHO (1998)	EGIR (1999)	ATPIII (2001)	AACE (2003)	IDF (2005)
Insulin resistance	IGT, IFG, T2DM, or lowered insulin Sensitivity ^a plus any 2 of the following	Plasma insulin >75th percentile plus any 2 of the following	None, but any 3 of the following 5 features	IGT or IFG plus any of the following based on the clinical judgment	None
Body weight	Men: waist-to-hip ratio >0.90; women: waist-to-hip ratio >0.85 and/or BMI > 30 kg/m ²	WC ≥94 cm in men or ≥80 cm in women	WC ≥102cm in men or ≥88 cm in women	BMI ≥ 25 kg/m ²	Increased WC (ethnic-specific) plus any 2 of the following
Lipids	TGs ≥150mg/dL and/or HDL <35 mg/dL in men or <39 mg/dL in women	TGs ≥150mg/dL and/or HDL <39 mg/dL in men or women	TGs ≥150mg/dL HDL <40mg/dL in men or <50mg/dL in women	TGs ≥150mg/dL and HDL <40mg/dL in men or <50mg/dL in women	TGs ≥150mg/dL or on TGs Rx. HDL <40mg/dL in men or <50mg/dL in women or on HDL Rx
BP	≥140/90mmHg	≥140/90mmHg or on hypertension Rx	≥130/85mm Hg	≥130/85mmHg	≥130mmHg systolic or ≥85 mm Hg diastolic or on hypertension Rx
Glucose	IGT, IFG, or T2DM	IGT or IFG (but not diabetes)	>110mg/dL (includes diabetes)	IGT or IFG (but not diabetes)	≥100mg/dL (includes diabetes) ^b

Other	Microalbuminuria: Urinary excretion rate of >20mg/min or albumin: creatinine ratio of >30mg/g.			Other features of insulin resistance ^c	
<p>^aIn insulin sensitivity measured under hyperinsulinaemic euglycaemic conditions, glucose uptake below lowest quartile for background population under investigation.</p> <p>^bIn 2003, the American Diabetes Association (ADA) changed the criteria for IFG tolerance from >110mg/dl to >100mg/dl.</p> <p>^cIncludes family history of T2DM, polycystic ovary syndrome, sedentary lifestyle, advancing age, and ethnic groups susceptible to T2DM.</p> <p>IFG: impaired fasting glucose; IGT: impaired glucose tolerance; BP: Blood pressure; Rx: receiving treatment; WC: waist circumference.</p>					

A major concern with the WHO and NCEP ATP III definitions of MetS has been their applicability to different ethnic groups, especially with regards to obesity cut-offs. The IDF have taken this factor into consideration and has proposed a set of criteria with ethnic-specific cut-off, recognising that the relationship between these values and the risk of T2DM and CVD differs amongst different ethnic groups (Kaur 2014).

Several studies have been conducted worldwide on the prevalence of MetS across different ethnic groups (Al-Lawati, Mohammed et al. 2003; Park, Zhu et al. 2003; Misra, Wasir et al. 2005; Shiwaku, Nogi et al. 2005). The incidence of MetS ranges from <10% to as much as 84%, depending on region, environment, gender, age, and ethnicity of the study population. According to the IDF, one-quarter of the world's population has MetS (Kaur 2014). People of Indian ethnicity have long been considered to be a "high-risk population" for both MetS and CVD, which has instigated a number of studies on Indians living in India and abroad (Tan, Ma et al. 2004; Misra, Wasir et al. 2005; Shah, Jonnalagadda et al. 2005).

In a large population-based survey of the multi-ethnic Malaysian population, an overall MetS prevalence of 27.5% was observed, with marked differences across ethnicities. Compared to ethnic Malays, the prevalence of MetS was 16% lower among Chinese and 31% higher among Indians (Rampal, Mahadeva et al. 2012).

Studies have shown increased thrombolysis in myocardial infarction (MI) time frame indicating impaired blood flow in coronary arteries even in the presence of angiographically normal coronary

arteries in MetS patients compared to patients without MetS, and is thought to play an important role in the pathogenesis of CAD (Aggarwal, Aggarwal et al. 2012).

Metabolic syndrome and T2DM often present together, thereby significantly increasing the risk of CAD. Individuals with MetS have a 5-fold increased risk for T2DM and twice the risk of developing CVD compared to those without the syndrome (Grundy 2008).

Parameter	Score
Age (years)	
<35	0
35-49	20
≥50	30
Abdominal obesity	
Waist <80 cm (female) , <90 (male)	0
Waist ≥ 80- 89 cm (female), ≥ 90 - 99 cm (male)	10
Waist ≥90 cm (female), ≥ 100 cm (male)	20
Physical activity	
Exercise (regular) + strenuous work	0
Exercise (regular) or strenuous work	20
No exercise and sedentary work	30
Family history	
No family history	0
Either parent	10
Both parents	20
Minimum score	0

Figure 2.4 Indian Diabetes Risk Score (Mohan, Sandeep et al. 2007)

The Indian Diabetes Risk Score (IDRS) makes use of clinical parameters such as age, WC, family history of diabetes, and physical activity to identify high-risk individuals (Figure 2.3). A score of ≥ 60 has been found to be useful in predicting MetS and cardiovascular disease (Mohan, Sandeep et al. 2007; Parikh and Mohan 2012).

(b) Tobacco

Cigarette smoking is considered one of the most important risk factors for vascular disease worldwide (Sadeghi, Adnani et al. 2013). Approximately 40% of all heart diseases have been related to smoking, and evidence suggests that smoking cessation is associated with a significant decrease in the risk of CAD (Teo, Ounpuu et al. 2006). Women who smoke have a similar risk of CAD as men, but more

than five times the risk of non-smoking women (Willett, Green et al. 1987; Klein and Nathan 2003). Passive smoking is an additional risk (Raupach, Schafer et al. 2006). Impaired vasodilatory function is an early manifestation of atherosclerosis. Cigarette smoking has shown to decrease nitric oxide (NO), the primary vasodilator of the endothelium. Cigarette smoking also increases inflammation, thrombosis, oxidation of LDL cholesterol, and oxidative stress, all of which impact atherosclerosis from endothelial dysfunction to acute clinical events. Smokers have a modified lipid profile, with significantly increased TGs, total and LDL cholesterol (Ambrose and Barua 2004).

(c) Hypertension

Hypertension is a chronic medical condition in which arterial BP is constantly elevated, and is defined by a systolic BP (SBP) of ≥ 140 mmHg or a diastolic BP (DBP) of ≥ 90 mmHg (Rosendorff, Lackland et al. 2015). The condition is a contributing factor in MetS, and one of the leading preventable causes of premature CAD (Kearney, Whelton et al. 2005). Hypertension is considered a major independent risk factor for CAD. Each increase of 20mmHg in SBP (or 10mmHg in DBP) doubles the risk of a fatal coronary event (Rosendorff, Black et al. 2007).

Hypertension causes an increased myocardial oxygen demand and decreased coronary blood flow, influencing vascular remodelling and atherosclerosis. The physical impact of hypertension can cause endothelial damage, characterised by an unfavourable balance between vasodilators (NO and prostaglandin E₁) and vasoconstrictors (endothelin and angiotensin II). This is enhanced by the accumulation of reactive oxygen species (ROS) and other inflammatory mediators which promote atherosclerosis, thrombosis, and vascular occlusion (Olafranye, Zizi et al. 2011).

Associated metabolic disorders such as T2DM, insulin resistance, and obesity are strongly correlated with hypertension. For example, obese individuals (BMI of ≥ 30 kg/m²) are more likely to be hypertensive than non-obese individuals. The mechanisms underlying the relationship between hypertension and obesity include sodium retention, activation of the renin-angiotensin-aldosterone system (RAAS), insulin resistance, and altered vascular function (Rosendorff, Lackland et al. 2015).

More than 80% of patients with T2DM develop hypertension, and approximately 20% of hypertensive patients develop diabetes. The pathogenesis of hypertension and T2DM is multifactorial, but recent evidence points toward the presence of an important component dependent on a low-grade inflammatory process (Savoia and Schiffrin 2007).

Activation of angiotensin II is a key component responsible for triggering vascular inflammation by inducing oxidative stress, resulting in up-regulation of pro-inflammatory transcription factors such as nuclear factor-kappa B (NF- κ B). This, in turn, upregulates the generation of inflammatory mediators as well as increases the breakdown of NO, leading to endothelial dysfunction and vascular injury contributing to the progression of atherogenesis. Inflammatory markers (mainly interleukin (IL)-6, tumour necrosis factor-alpha (TNF- α), CRP, chemokines and adhesion molecules) are increased in patients with hypertension and metabolic disorders, and predict the development of CVD. (Savoia and Schiffrin 2007). In addition to angiotensin II, endothelin-1 also plays an important role in mediating chronic inflammation in the vascular wall by similar mechanisms (Viridis and Schiffrin 2003; Li and Chen 2005).

(d) Dyslipidaemia

Dyslipidaemia refers to an altered lipid profile characterised by increased levels of total cholesterol, LDL, TGs, and decreased HDL (Achari and Thakur 2004). High levels of LDL in circulation, in the presence of other risk factors including hypertension, T2DM, and smoking, causes oxidative modification of Lp(a). This induces the expression of adhesion molecules, chemokines, proinflammatory cytokines, and other inflammatory mediators. Apart from LDL, other lipoprotein molecules such as very low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL) are also susceptible to oxidative modification and therefore have atherogenic potential. Accumulation of lipid in the intima of coronary arteries form the necrotic core of the atherosclerotic plaque (Libby and Theroux 2005). High density lipoprotein serves a protective function against atherosclerosis, responsible for reverse cholesterol transport (RCT) and transport of antioxidant enzymes which degrade oxidised lipids and neutralise their proinflammatory effects (Libby, Ridker et al. 2002).

(e) Diet, obesity, and physical inactivity

An unhealthy diet comprising of high-fat foods, red meat, refined grains, and cholesterol-rich meals increases the risk of heart disease (Fung, Rimm et al. 2001). Interestingly, a study showed an inverse association between fruit and vegetable intake and the development of CVD events such as acute plaque rupture (Ignarro, Balestrieri et al. 2007).

Obesity may be due to genetic inheritance or a result of unhealthy eating and sedentary lifestyle (Poirier, Giles et al. 2006), and is strongly associated with hypertension, endothelial dysfunction, and CAD (Lavie, Milani et al. 2009). Obesity is a predisposing factor for insulin resistance and diabetes, and contributes to dyslipidaemia. High levels of free fatty acids enter the liver and stimulate the production of triglyceride-rich VLDL. Elevated levels of VLDL can reduce HDL levels by enhancing

conversion of HDL to VLDL by cholesteryl ester transfer protein. Adipose tissue harbours resident macrophages which produce TNF- α and IL-6, which promote inflammation (Libby, Ridker et al. 2002; Galkina and Ley 2009).

Physical inactivity has been linked to CVD, whereas moderate exercise has been shown to prevent the incidence of atherosclerosis and CVD. Physical activity in combination with weight loss prevents and treats many established CAD risk factors such as hypertension, insulin resistance, increased triglycerides, LDL cholesterol and obesity (Thompson, Buchner et al. 2003).

Coronary artery disease is a complex multifactorial disease characterised by the interaction of genetic, epigenetic, environmental, and lifestyle-associated factors. The risk factors above often occur in clusters and may build on one another, putting an individual at an even greater risk of CAD. In some cases, CAD develops without any of the classical risk factors. Studies have identified factors that may be responsible for this phenomenon, and classified them as “novel” risk factors (Wilson 2004).

2.3.3 “Novel” risk factors

(a) Excess homocysteine

Elevated plasma homocysteine (Hcy) has been associated with risk of CAD. Levels of Hcy are determined by lifestyle as well as genetic predisposition. The methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism is one of the strongest genetic predictors of Hcy (Mehlig, Leander et al. 2013). Increased Hcy can block the synthesis of NO, allowing the build up of plaque.

Stühlinger *et al.* showed that Hcy post-transcriptionally inhibits dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that degrades asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase. This in turn causes the accumulation of ADMA and inhibition of NO synthesis (Stühlinger, Tsao et al. 2001). In addition, the sulfhydryl (SH) group of Hcy is oxidised to a disulphide bond in a reaction coupled to the formation of ROS such as hydrogen peroxide and superoxide. This increases the oxidative degradation of NO, inhibiting endothelium-dependent NO-mediated dilation and favouring endothelial dysfunction (Carmel and Jacobsen 2001; Omae, Nagaoka et al. 2013).

The association between Hcy and CAD is furthermore due to promotion of smooth muscle cells into the intima, enhanced platelet aggregation, increased binding of Lp(a) to fibrin, production of free radicals, and formation of oxidised LDL (oxLDL) (Coffey, Crowder et al. 2003).

The beneficial effects of Hcy-lowering therapy have been investigated. Although a large number of placebo-controlled clinical trials have been carried out to evaluate the efficacy of Hcy-lowering drugs, these interventions have not been proven to prevent the occurrence of cardiovascular events (Debrececi and Debrececi 2012).

(b) C-reactive protein

C-reactive protein is a plasma protein synthesised in the liver and is a sensitive marker of systemic inflammation. Experimental and clinical studies provide evidence of a direct contribution of CRP towards the pathogenesis of atherosclerosis. C-reactive protein binds to damaged cells and activates the complement system suggesting a potential proatherogenic role in foam cell formation and progression of atherosclerotic lesions. It also binds to degraded LDL and colocalizes with these particles in early atherosclerotic lesions. C-reactive protein is a powerful stimulator of tissue factor production by monocytes and this effect is increased in the presence of other inflammatory mediators (Arici and Walls 2001; Hage and Szalai 2007; The Emerging Risk Factors 2010).

(c) Lipoprotein(a)

Lipoprotein(a) is a LDL-like molecule that consists of an apolipoprotein B-100 (apo B-100) particle attached by a disulphide bridge to apolipoprotein A (apo A). Lipoprotein(a) contributes to atherosclerosis by causing endothelial dysfunction, promoting the proliferation of smooth muscle cells into the intima, and inducing the action of proinflammatory mediators. Furthermore, Lp(a) can undergo oxidative modification and scavenger uptake, resulting in foam cell formation (Malaguarnera, Vacante et al. 2013).

2.4 Pathogenesis of coronary artery disease: Atherosclerosis – Roles of inflammation, endothelial perturbation and lipids

Dyslipidaemia constitutes the major underlying basis of CAD. Increased lipid levels promote their penetration of arterial walls. Low-density lipoprotein is the primary atherogenic lipid.

The early stages of atherosclerosis is characterised by endothelial dysfunction which may be triggered by several factors including oxLDL, free radicals, elevated Hcy, and chronic systemic infection (Figure 2.5 A.) (Davis 2005; Menghini, Stöhr et al. 2014).

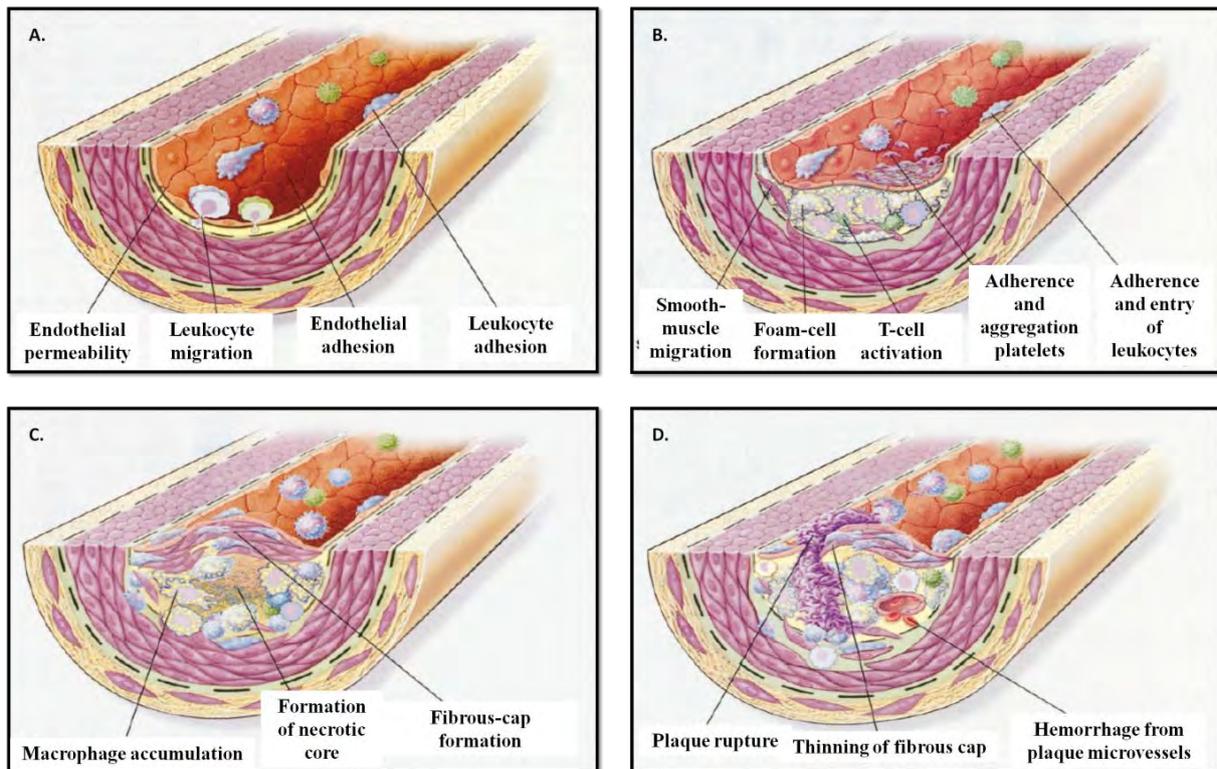


Figure 2.5 An overview of atherosclerosis in the coronary artery **A.** Endothelial dysfunction triggered mainly by exposure of the endothelium to oxLDL molecules attracts T-lymphocytes, monocytes, and platelets to the site of injury. **B.** LDL particles are engulfed by monocytes which thereafter transform into foam cells. Smooth muscle cells migrate from the media, and fatty streaks are formed. **C.** Accumulation of fatty streaks leads to a fibrous cap. The initial macrophages and monocytes begin to die and form a necrotic core covered by the fibrous cap. **D.** As the cap increases in size, the artery wall begins to expand until the plaque bulges into the vessel lumen. The fibrous cap eventually thins and ruptures, releasing the contents into the vessel lumen and resulting in thrombus formation. A large enough thrombus can cause luminal occlusion, causing myocardial infarction or stroke (Davis 2005).

In an attempt to repair itself, the endothelium triggers the recruitment of T-lymphocytes, monocytes and platelets to the site of injury. When this reparative process fails, the permeability of the endothelium increases and lymphocytes and monocytes migrate deep into the intima, attracting LDL particles to the site. Activation of monocytes leads to cytokine-mediated progression of

atherosclerosis and oxidation of LDL (Figure 2.5 A.) (Crowther 2005; Davis 2005; Nazari-Jahantigh, Egea et al. 2014).

Monocytes differentiate into macrophages and upregulate scavenger receptors and Toll-like receptors (TLRs) (Galkina and Ley 2009). Oxidised LDL is atherogenic and chemotactic for monocyte-macrophages. The scavenger receptors mediate the uptake of oxLDL by the macrophages and results in foam cell formation, a process recently shown to be modulated by epigenetic mechanisms (Menghini, Stöhr et al. 2014). Macrophages become less mobile after the uptake of oxLDL, thereby promoting the accumulation of these lipid-rich cells in the intima. Toll-like receptor signalling leads to the release of cytokines, proteases, and vasoactive molecules (Figure 2.5 B.) (Crowther 2005; Hansson, Robertson et al. 2006; Nazari-Jahantigh, Egea et al. 2014).

Many mediators of inflammation have been implicated in driving the process of atherosclerosis. These mediators include, but are not limited to, TNF- α and TNF-beta (β), interleukin IL-1 β , IL-6, monocyte-colony stimulating factor (M-CSF), monocyte chemoattractant protein (MCP)-1, and IL-18 (Crowther 2005). The functions of these inflammatory mediators range from angiogenesis to foam cell formation.

Smooth muscle cells begin migrating from the media and accumulation of lipid-laden foam cells form fatty streaks, the earliest visible lesions of atherosclerosis (Crowther 2005). More lymphocytes are recruited to the lesion, thus contributing to the growing pool of effector cells that perpetuate the inflammatory response (Figure 2.5 C.) (Chilton 2004).

Progression of fatty streaks leads to a further attempt of endothelial repair, and a fibrous cap consisting of smooth muscle and collagen develops. The monocytes and macrophages from the original reaction die, resulting in the formation of a necrotic core covered by the fibrous cap. The fibrous cap and necrotic core, known as the atherosclerotic plaque, is the hallmark of established atherosclerosis (Figure 2.5 C.) (Crowther 2005).

Accumulation of lipid is required for the development and enlargement of the atherosclerotic plaque. An unstable plaque can rupture, releasing lipid fragments and cellular debris into the lumen and resulting in thrombus formation (Davis 2005). If large enough, the thrombus causes luminal occlusion, leading to MI or stroke (Figure 2.5 D.) (Davis 2005).

2.5 Genetic variation in coronary artery disease

Substantial progress has been made in understanding the genetic basis of human diseases since the completion of the human genome sequence. The genetic basis of CAD is especially important given that this is the leading cause of mortality worldwide. However, with the complexity of CAD, comes along several challenges – ranging from phenotypic and genetic heterogeneity to gene-gene and gene-environment interactions (Table 2.2) (Kullo and Ding 2007).

Table 2.2 Challenges in identifying genetic determinants of coronary heart disease *reproduced from Kullo and Ding 2007* (Kullo and Ding 2007)

Challenge	Comment
Phenotypic heterogeneity	CHD can manifest as several clinical phenotypes, including chronic stable angina, acute coronary syndrome, MI, sudden cardiac death, and history of coronary revascularization. Measures of coronary atherosclerotic burden such as coronary artery calcium or angiographic coronary artery disease are objective and quantitative, unlike the dichotomous characterization (presence or absence) of a history of a cardiovascular event. Atherosclerotic disease burden and cardiovascular events such as myocardial infarction are distinct phenotypes, however, the latter being related more to plaque instability and rupture rather than plaque burden.
Genetic heterogeneity	Genetic heterogeneity is likely, given the multiple causal pathways that lead to CHD. For example, low plasma levels of HDL cholesterol can result from variation in genes from multiple metabolic pathways. Similarly, the transition from a stable coronary atherosclerotic plaque to an unstable inflamed plaque could be the result of genetic variation in multiple genes that participate in the inflammatory cascade and matrix degradation.
Small gene effects	A single genetic variant can constitute only a small proportion (e.g. 1–2%) of the total genetic contribution towards complex disease phenotypes, and an OR of 1.1–1.5 is typical for a susceptibility variant of a candidate gene (Risch 2000). Uncovering such small effects requires large sample sizes, and recognition of this fact has motivated assembly of the so-called Biobanks in several European countries, including Iceland, England and Estonia (Kaiser 2002)
Gene-gene and gene-environment	Identification of gene-gene and gene-environment interactions is essential for identification of genes responsible for complex diseases, and will require large

interactions	sample sizes and adequate computational resources.
Rare variants causing complex disease	Most current genetic epidemiology studies of complex diseases such as CHD assume that common variants (i.e. minor allele frequency $\geq 5\%$) account for much of the susceptibility to the disease. Both common and rare variants, however, probably influence CHD susceptibility. To uncover rare variants that influence susceptibility to CHD, resequencing or very large sample sizes will be required.
Abbreviation: CHD, coronary heart disease.	

Genetic variation occurs as a result of structural change(s) in the gene sequence due to modification of deoxyribonucleic acid (DNA) bases, producing different forms of the gene known as polymorphisms (Dzimiri, Wang et al. 2012). A single nucleotide polymorphism (SNP) is the most common type of genetic variation in the human genome (Xu, Zhu et al. 2008). As the name suggests, a SNP is a single base pair (bp) change in the DNA sequence. Each SNP is characterised by two alleles, together known as a genotype, with each allele existing on each homologous chromosome (Lanktree 2008). The frequency of SNPs within a particular gene varies from one population to another, and various SNPs have been associated with disease.

2.5.1 Association studies: Identifying genes related to coronary artery disease – The candidate gene approach

Association studies compare allele frequencies in cases and controls to assess the contribution of genetic variants to phenotypes of interest. Genetic variants occurring more frequently in cases than controls are considered to be associated with disease risk (Roberts and Stewart 2012).

Genome-wide association studies (GWAS) search the entire genome for genetic variation. In contrast, the candidate gene approach focuses on association between a specific gene of interest and disease (Bampali, Mouzarou et al. 2014).

Single nucleotide polymorphisms implicated in CAD are frequently found in genes controlling various mechanisms (e.g. lipid and glucose metabolism, inflammation, BP regulation, and vascular contractility) (Dzimiri, Wang et al. 2012).

2.6 Epigenetics and coronary artery disease

Epigenetics is a term used to refer to mechanisms that regulate gene expression that do not involve alteration in DNA sequence. The field of epigenetic research is relatively new but rapidly growing, and may provide novel insight into the complex mechanisms underlying CAD. A number of international initiatives have commenced, including the Human Epigenome Project (HEP) in 2003 and the International Human Epigenome Consortium (IHEC) in 2010 (Webster, Yan et al. 2013).

Three areas of epigenetics exist: DNA modification, histone modification, and ribonucleic acid (RNA)-based mechanisms (Pons, de Vries et al. 2009; Webster, Yan et al. 2013). The most investigated epigenetic mechanisms are DNA methylation and histone modification. RNA-based mechanisms such as microRNA (miRNA; miR) involvement are the newest branch of epigenetics.

2.6.1 DNA Modification

DNA methylation refers to the covalent methylation of cytosines located in CG dinucleotides (CpGs) catalyzed by DNA methyltransferases (DNMT). CpG sites are relatively rare in the genome but occur more regularly at the promoter regions of genes, and are collectively known as CpG islands (van Eijk, de Jong et al. 2012). DNA methylation controls gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factor(s) to DNA. Increased methylation of CpG islands at 5' end of a gene is associated with gene repression. The mechanisms for gene repression include interference with transcription factor binding or through the recruitment of repressors such as histone deacetylases (HDAC) (van Eijk, de Jong et al. 2012; Moore, Le et al. 2013).

S-adenosyl-methionine (AdoMet; SAM) is the main source of methyl groups for methylation reactions. After the transfer of methyl groups, S-adenosyl-Hcy (AdoHcy; SAH) is formed which can be hydrolysed to Hcy and adenosine. If Hcy accumulates (a well-known risk factor for CAD), AdoHcy also accumulates and increased AdoHcy is known to inhibit methyltransferases. Therefore Hcy levels may regulate global DNA methylation via AdoHcy (Handy, Castro et al. 2011).

Methylenetetrahydrofolate reductase is an enzyme that catalyses the reduction of 5',10'-methylenetetrahydrofolate to 5'-methylenetetrahydrofolate; a major form of folate in plasma, and a carbon donor for the remethylation of Hcy to methionine (Kluijtmans and Whitehead 2001; Vasisht, Gulati et al. 2002). The enzyme is thus responsible for reducing levels of Hcy in the plasma. The TT genotype of the common MTHFR C677T SNP (rs1801133) has been associated with increased Hcy and diminished genomic DNA methylation (Stern, Mason et al. 2000).

2.6.2 Histone Modification

Genomic DNA is highly organised, and packed with histones to form chromatin. The nucleosome is the basic building block of chromatin, and consists of ~147bp of DNA wrapped around an octamer of histone proteins. Although core histones are tightly packed, their NH₂-terminals can undergo modification by histone-modifying enzymes (Ling and Groop 2009).

Histone modifications include methylation, phosphorylation, acetylation, and ubiquitination and are essential determinants of DNA transcription, replication, recombination, and chromosomal organisation (Schnabel, Baccarelli et al. 2012).

The addition of acetyl groups by histone acetyl transferases (HAT) is thought to interfere with histone-DNA interactions, and allowing increased transcription factor access to DNA by “loosening” chromatin structure. Histone deacetylases (HDAC) remove acetyl groups and inhibit transcription factor access and transcription of genes (Mack 2008).

Sirtuin (SIRT) 1, a class III HDAC, has been identified as a candidate molecule affecting the epigenetic mechanisms of CVD. Previous studies have shown that some SIRT 1 SNPs are associated with inflammation, BMI, diabetes, BP, and dyslipidaemia, all of which are well-established risk factors for CAD (Finkel, Deng et al. 2009; Horio, Hayashi et al. 2011; Cui, Wang et al. 2012; Houtkooper, Pirinen et al. 2012).

2.6.3 Micro RNAs

MicroRNAs are a class of small (~22 nucleotides in length), endogenous, single-stranded, non-coding RNA molecules that act by degrading or inhibiting the translation of messenger RNAs (mRNAs) (Jamaluddin, Weakly et al. 2011). These highly conserved molecules exert their function at post-transcriptional level as negative regulators of gene expression (Jamaluddin, Weakly et al. 2011; Mittal, Gangwar et al. 2011; Raitoharju, Lyytikainen et al. 2011). Since their discovery in 1993 (Lee, Feinbaum et al. 1993), over 2500 human miRNAs have been described (Kozomara and Griffiths-Jones 2014).

Before publication of their discovery, experimentally confirmed miRNAs are given a name under a standard nomenclature system (Table 2.3)

Table 2.3 Primary features of the miRNA nomenclature system *adapted from (Wright and Bruford 2011; Kozomara and Griffiths-Jones 2014)*

miRNA	Nomenclature	Example
miRNA	prefix “miR” is followed by a dash and a number, the latter indicates the order of naming	miR-146 was named and likely discovered before miR-499
Mature form of the miRNA	Capitalised prefix “miR”	miR-499
Precursor-miRNA and primary-miRNA	Referred to as pre- and pri- respectively, followed by an uncapitalised “mir”	pre-mir-155 and pri-mir-155
Closely related miRNAs	miRNAs that have an almost identical sequence (differing by one or two nucleotides) are given an additional lower case lettered suffix	miR-146a and miR-146b
Sequences derived from the 5’ and 3’ arms of the hairpin precursor	Are given an additional -5p and -3p respectively	miR-100-5p and miR-100-3p
miRNA species	A three or four letter species prefix and numeric suffix	hsa-miR-125 (<i>Homo sapiens</i>) mmu-miR-125 (<i>Mus musculus</i>)

2.6.3.1 MicroRNA biogenesis and processing

MicroRNAs are derived from longer primary RNA transcripts (pri-miRNAs). Processing begins in the nucleus, where the RNase III endonuclease Drosha and its binding partner DiGeorge syndrome chromosomal (or critical) gene 8 (DGCR8), cleaves the pri-miRNA into shorter (~70-85 nucleotides) precursor (pre-) miRNAs that consist of a stem-loop “hairpin-like” structure (Jamaluddin, Weakly et al. 2011). Precursor-miRNAs are then exported into the cytoplasm by exportin 5, and cleaved by DICER and its binding partner trans-activation response (TAR) binding protein (TARBP) into a miRNA duplex which is composed of a mature miRNA sequence (~22 nucleotides) and its complementary sequence miR*. The miRNA is loaded onto the RNA-induced silencing complex (RISC), and interacts with the 3’ untranslated region (UTR) of its target mRNA to control gene expression (Jamaluddin, Weakly et al. 2011) (Figure 2.6).

The seed sequence of the miRNA is a conserved region at positions 2-8 from the miRNA 5'-end and is important for target recognition. Binding of the miRNA and the target mRNA 3' UTR determines the mechanism of mRNA-mediated gene regulation (Liu, Fortin et al. 2008; Lowery, Miller et al. 2008; Jamaluddin, Weakly et al. 2011; Raitoharju, Lyytikainen et al. 2011; Rusca and Monticelli 2011; Goldring 2012). Translation repression occurs when there is imperfect base pairing (mostly in animals) and perfect base pairing results in mRNA cleavage and degradation (mostly in plants) (Bartel 2004).

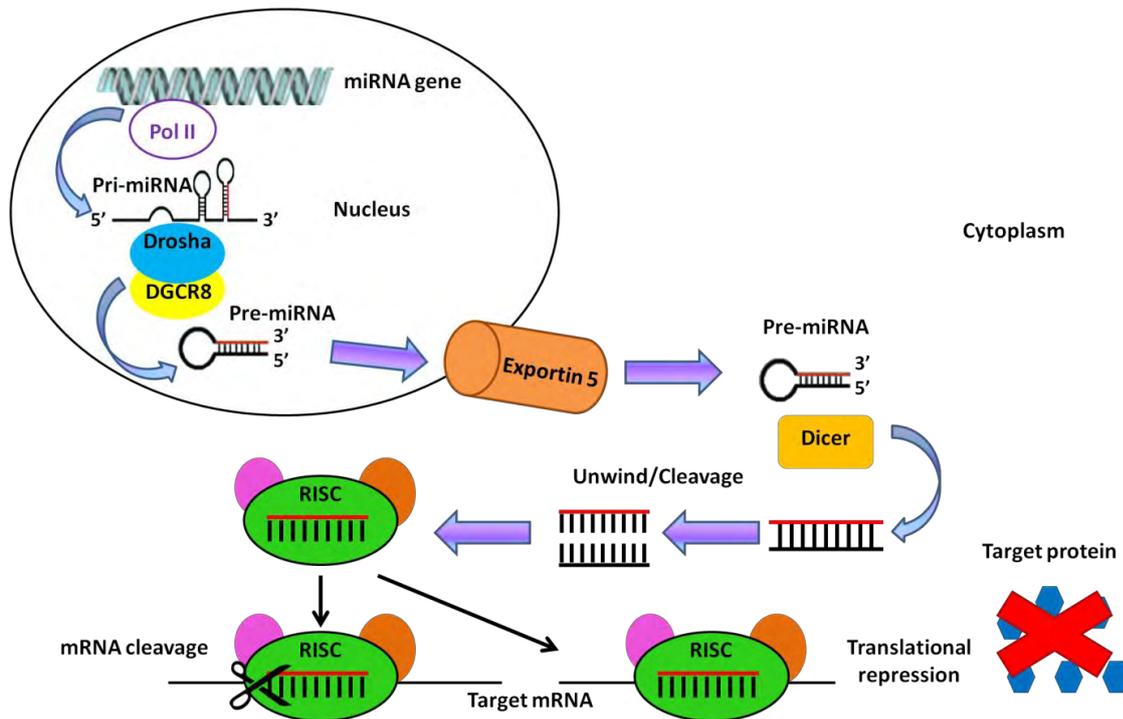


Figure 2.6 Biogenesis and processing of miRNAs (Author's own work)

A single miRNA has target sites in hundreds of different genes, and more than 60% of protein-coding genes are conserved targets of miRNAs (Dai and Ahmed 2011). MicroRNAs are involved in almost all biological processes, including development, cell differentiation, proliferation, and apoptosis (Xu, Zhu et al. 2008).

Aberrant miRNA regulation has been implicated in several disease conditions (Li, Chen et al. 2010). Recent studies reveal an important role of miRNAs in vascular biology and CVD, including CAD. Numerous miRNAs are highly expressed in the vasculature, and diseased vessels show dysregulated expression of miRNAs (Jamaluddin, Weakly et al. 2011; Stefano, Zaccagnini et al. 2011).

2.6.3.2 microRNAs in coronary artery disease

The discovery of miRNAs as promising new biomarkers for CAD has ignited great expectations (Figure 2.7), and is being investigated as potential targets for therapeutic intervention. Having fulfilled several requirements of an ideal biomarker (stability in circulation, tissue- and pathology-specific regulation as well as high sensitivity and specificity), miRNAs may even exceed protein-based biomarkers (Schulte and Zeller 2015).

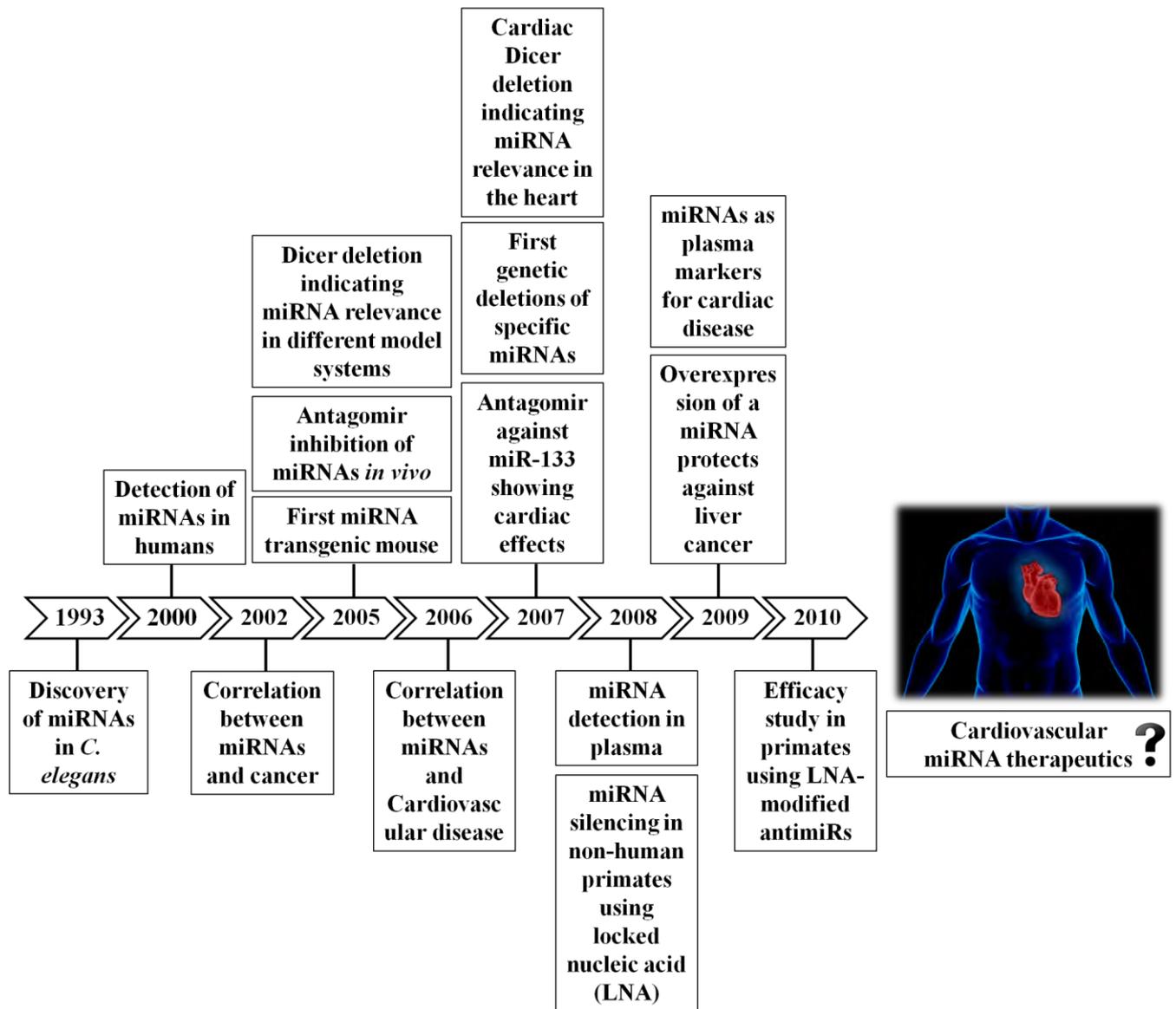


Figure 2.7 Breakthrough discoveries in miRNA biology. Time line indicating seminal discoveries in miRNA biology with special focus on the cardiovascular field *adapted from van Rooji 2011* (van Rooji 2011)

Case-control studies have shown dysregulated miRNA levels in CAD patients compared with healthy controls (Contu, Latronico et al. 2010; Creemers, Tijssen et al. 2010; Fichtlscherer, De Rosa et al.

2010). The study of miRNAs in CVD is an emerging field of research and much is yet to be discovered.

2.6.3.3 Single nucleotide polymorphisms in microRNA genes

The binding of miRNA to mRNA is essential for regulating mRNA and protein expression. Given that miRNAs are so small in size and the critical role played by the seed sequence in miRNA-mRNA binding, a single nucleotide change in sequence could have a major consequence on miRNA-mRNA interaction and downstream expression (Jin and Lee 2013). Several miRNA SNPs were shown to affect miRNA expression and/or miRNA-mRNA interaction, and have been associated with human diseases (Chen, Song et al. 2008; Ardekani and Naeini 2010; Li, Chen et al. 2010). Single nucleotide polymorphisms in inflammatory-associated miRNAs such as miR-499 and miR-146a are of particular interest due to their implication in chronic inflammatory diseases.

(a) miR-499 and rs3746444

MicroRNA-499 acts by targeting several cytokines that play a role in the process of atherosclerosis. Interleukin-2, IL-2 β , IL-6, IL-17, IL-17R β , IL-18, IL-21, and IL-23a are well-known targets of miR-499 (Yang, Chen et al. 2012). A common miR-499 SNP, rs3746444, has been implicated in various inflammatory diseases (Li, Wang et al. 2011; Li, Gao et al. 2011; Hashemi, Eskandari-Nasab et al. 2013).

The miR-499 A/G SNP, located within the seed sequence of miR-499, results in a change from an A:U pair to a G:U mismatch (Figure 2.8). The variant (G) allele has a less stable secondary structure than the ancestral (A) allele and affects the binding to target mRNAs (Akkız, Bayram et al. 2011).

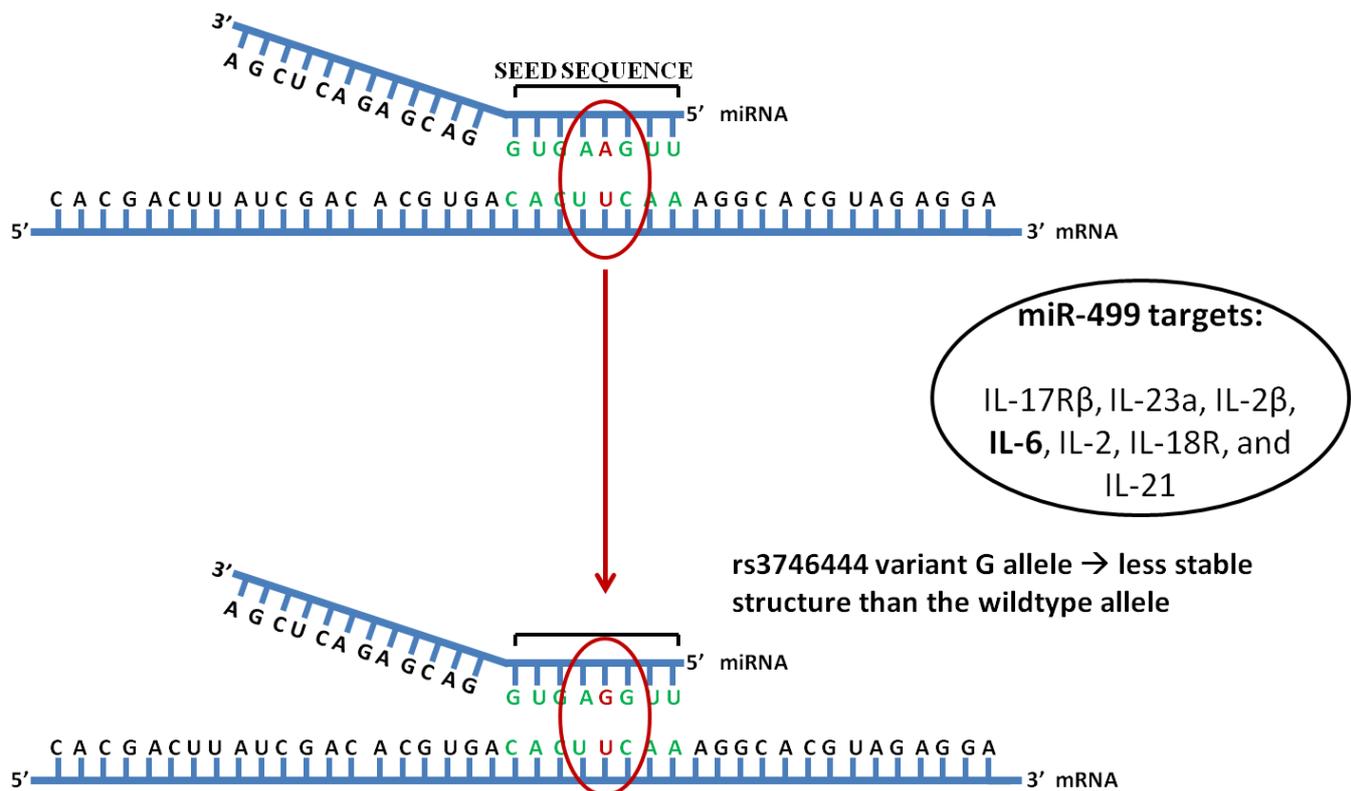


Figure 2.8 A common miR-499 SNP (rs3746444) located within the seed sequence results in a change from A-to-G (Author's own work)

In a Chinese Han population, Yang *et al.* found that rheumatoid arthritis (RA) patients who were carriers of the heterozygote rs3746444 variant had significantly higher erythrocyte sedimentation rate (ESR) and IL-6 compared to those with the homozygous wildtype. Their observations indicated that the SNP could be important in affecting the inflammatory response in RA patients (Yang, Chen et al. 2012). These findings were supported by Hashemi *et al.* and El-Shal *et al.* who found that the variant allele was a risk factor for RA in Iranian and Egyptian populations respectively (El-Shal, Aly et al. 2013; Hashemi, Eskandari-Nasab et al. 2013). Interestingly, The heterozygous variant of rs3746444 was associated with a decreased risk for chronic obstructive pulmonary disease (COPD) (Li, Gao et al. 2011), but no association was found with systemic lupus erythematosus (SLE) in Chinese populations (Zhang, Yang et al. 2011). All four of these studies also investigated the miR-146a SNP, rs2910164, with no significant results being observed.

Various genetic association studies have explored the role of rs3746444 in cancers including hepatocellular carcinoma (Akkız, Bayram et al. 2011), breast cancer (Catucci, Yang et al. 2010), and prostate cancer (George, Gangwar et al. 2011). Due to mixed and inconclusive results, a recent meta-

analysis involving 7974 cases and 9404 controls was done to summarise and clarify the association between rs3746444 and cancer risk. The results showed that the miR-499 A/G SNP was associated with increased risk of cancer. Further subgroup analysis by ethnicity showed that association between rs3746444 and cancer susceptibility was significant in Asians, but not in Caucasians (Sun, Li et al. 2014).

To date, there have been only two published studies on the miR-499 rs3746444 with regards to CAD (Zhi, Wang et al. 2012; Xiong, Cho et al. 2014). Recently, Zhi *et al* found that the GG genotype was associated with a 3.23-fold increased risk of CAD (Zhi, Wang et al. 2012). In contrast, Xiong *et al* found no association between rs3746444 and CAD in a Chinese Han population (Xiong, Cho et al. 2014).

(b) miR-146a and rs2910164

MicroRNA-146a acts by targeting IL-1 receptor associated kinase (IRAK)-1 and TNF- α receptor associated factor (TRAF)-6, which act downstream of toll-like and IL-1 receptors (TIR) signalling pathway (Figure 2.9). This pathway occurs in a NF- κ B-dependent manner (Chatzikyriakidou, Voulgari et al. 2010; Li, Chen et al. 2010), and is involved in regulating inflammation (Roldán, Arroyo et al. 2014).

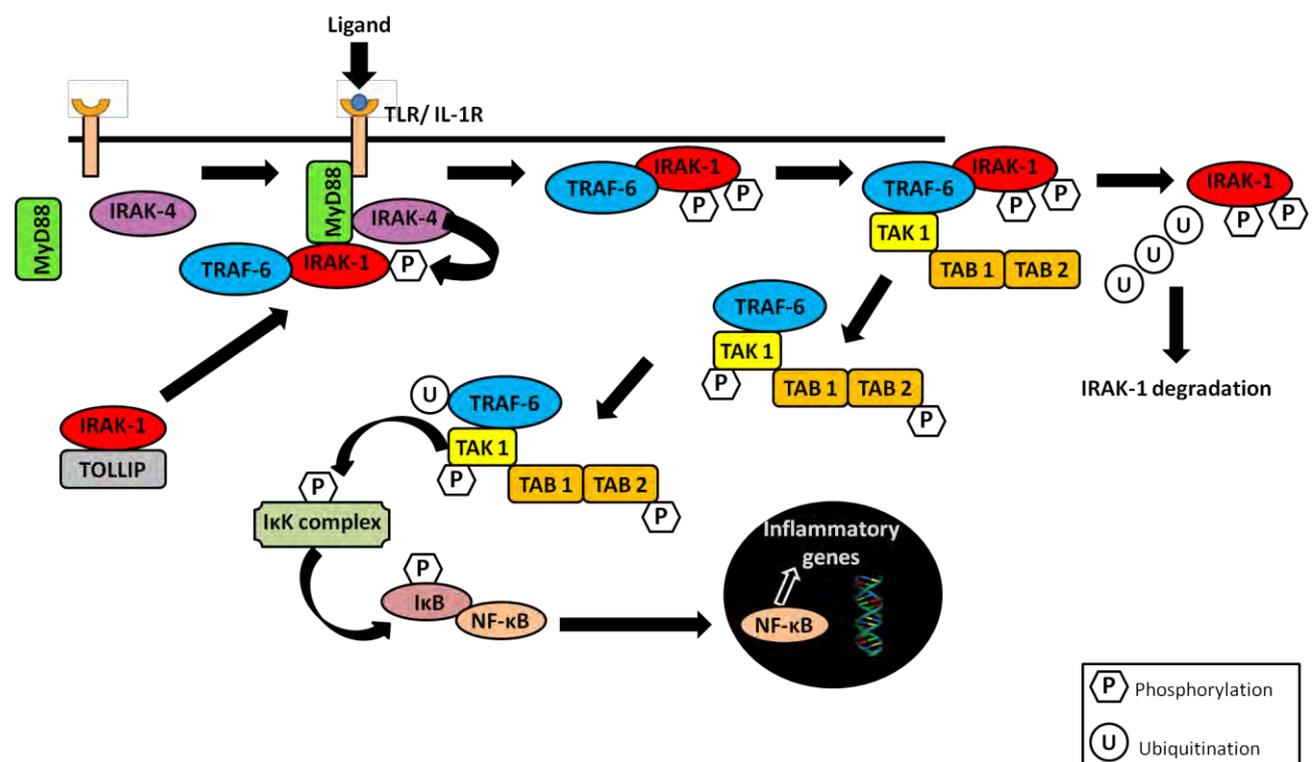


Figure 2.9 The role of IRAK-1 and TRAF-6 (targets of miR-146a) in the TIR signalling pathway (Author's own work)

Under conditions of hyperlipidaemia, TLRs participate in regulating atherosclerosis and inducing the production of proinflammatory cytokines (Galkina and Ley 2009). Prior to activation of the receptor, myeloid differentiation primary response gene 88 (MyD88), TRAF-1 and IRAK-4 are found in the cytosol. IRAK-1 is found in association with toll interacting protein (TOLLIP), an inhibitory adaptor protein (Gottipati, Rao et al. 2008). Upon binding of the ligand to the TIR, MyD88 is rapidly recruited to the receptor via interaction of the death domains of both molecules (Takeda and Akira 2004; Flannery and Bowie 2010). IRAK-1 is recruited to the receptor complex and binds to MyD88, which brings it closer to IRAK-4 (O'Neill 2008). This results in the phosphorylation of IRAK-1 by IRAK-4, resulting in the activation of IRAK-1 kinase activity and formation of hyper-phosphorylated IRAK-1 by auto-phosphorylation.

MyD88 and TOLLIP only bind to non-phosphorylated IRAK-1, thus upon phosphorylation IRAK-1 is released from the receptor complex and binds to TRAF-6 (Flannery and Bowie 2010). IRAK-1 and TRAF-6 interact via multiple domains, including their death domains and C-terminals (Gottipati, Rao et al. 2008). The IRAK-1/TRAF-6 complex dissociates from the receptor complex and interacts with a performed complex made up of transforming growth factor-beta (TGF- β)-activated kinase (TAK) 1, TGF- β -activated kinase-binding protein (TAB) 1, and TAB2 (Janssens and Beyaert 2003). This leads to the phosphorylation of TAB2 and TAK1, and further translocation into the cytosol.

IRAK-1 remains at the membrane and is degraded by a ubiquitin-dependent mechanism, while TRAF-6, TAK1, TAB1 and TAB2 associate with ubiquitin ligases (Muroi and Tanamoto 2012). This causes the ubiquitination of TRAF-6, an important step required for activating the kinase activity of TAK1. The inhibitor of kappa B (I κ K) complex (consisting of I κ K- α , I κ K- β , and I κ K- γ) is phosphorylated by TAK1. The complex in turn phosphorylates inhibitor of NF- κ B kinase subunit beta (I κ B), resulting in its degradation. This allows the translocation of NF- κ B into the nucleus and subsequent binding to specific promoter sequences, activating inflammatory genes (Chung, Park et al. 2002; Perry, Moschos et al. 2008).

Several SNPs have been identified in the miR-146a gene. A common SNP; rs2910164, has been widely studied in RA, tuberculosis (TB), and several cancers (Chatzikyriakidou, Voulgari et al. 2010; Li, Wang et al. 2011; Yue, Wang et al. 2011). Few studies have focused on CAD, especially in the Indian population.

The G-to-C SNP (rs2910164) has been identified in the crucial seed region of the miR-146a gene (Jazdzewski, Liyanarachchi et al. 2009) (Figure 2.10), causing a change from a G:U pair to a C:U mismatch (Hung, Chang et al. 2012). This SNP seems to affect the expression of mature miR-146a, however whether the G- or C-variant causes a decrease/increase in mature miR-146a remains controversial (Chatzikyriakidou, Voulgari et al. 2010; Yue, Wang et al. 2011; Hung, Chang et al. 2012).

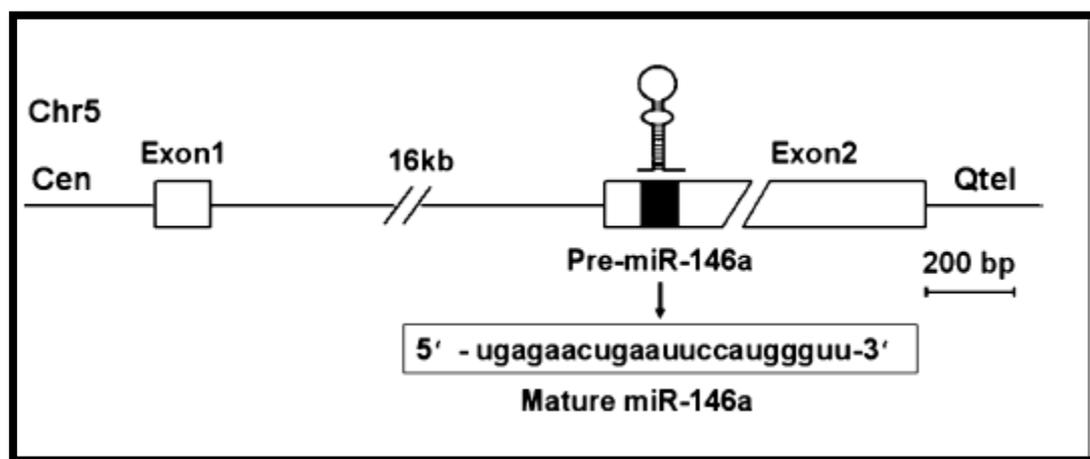


Figure 2.10 Schematic representation of the miR-146a loci and the sequence of mature miR-146a on human chromosome 5 (Li, Chen et al. 2010)

Although observing no association between rs2910164 and RA, Chatzikyriakidou *et al.* reported increased miR-146a expression in synovial fibroblasts, synovial tissue, and PBMCs from RA patients (Chatzikyriakidou, Voulgari et al. 2010). A similar finding was observed by Pauley *et al.* who found an increased expression of miR-146a in PBMCs from RA patients, coupled with similar expression of IRAK-1 and TRAF-6 in both patients and controls despite the increased expression of miR-146a in RA patients (Pauley, Satoh et al. 2008).

In contrast to increased expression of miR-146a in RA patients, there have been reports of decreased miR-146a expression in patients with SLE. A recent study reported that miR-146a is intrinsically underexpressed in SLE patients, and miR-146a levels correlated negatively with SLE activity (Tang, Luo et al. 2009). This is interesting as both RA and SLE are autoimmune diseases. The precise

mechanism for the difference in miR-146a expression among the different diseases remains unknown. The overall cytokine profiles in these disease may account for these patterns (Li, Chen et al. 2010).

The effect of rs2910164 on miR-146a expression is heterogeneous across different cancers (Hung, Chang et al. 2012). Yue *et al.* showed that the G-variant is associated with decreased expression of mature miR-146a and increased risk for cervical cancer (Yue, Wang et al. 2011), while Shen *et al.* demonstrated that the C-variant caused increased expression of mature mir-146a and increased the risk of an early onset familial breast and ovarian cancers (Shen, Ambrosone et al. 2008). Hung *et al.* observed increased expression of miR-146a in oral squamous cell carcinoma (OSCC) patients with the rs2910164 C variant (Hung, Chang et al. 2012). Xu *et al.* showed that the G-variant results in an increase in mature miR-146a, and a two-fold increase in susceptibility to liver cancer (Xu, Zhu et al. 2008). The C-variant caused a decrease in miR-146a, and decreased risk of prostate carcinoma (Xu, Feng et al. 2010). In 2008, Jazdzewski et al. observed a 1.8-fold decrease in miR-146a with the C-variant compared to the G-variant, and that the GC genotype is associated with an increased risk of papillary thyroid carcinoma (Jazdzewski, Murray et al. 2008).

In 2011, Zilahi et al. measured the expression of miR-146a, and its target genes, IRAK-1 and TRAF-6 in the PBMCs of patients with Sjögren's syndrome and healthy controls (Zilahi, Tarr et al. 2011). Their results showed that while both miR-146a and TRAF-6 were significantly overexpressed in patients with Sjögren's syndrome, the expression of IRAK-1 was significantly decreased. They suggested that as IRAK-1 is regarded an important gene in the pathogenesis of SLE, TRAF-6 could be a biomarker specific to Sjögren's syndrome.

Li et al. investigated the miR-146a rs2910164 and susceptibility to TB in the Chinese Tibetan and Han population (Li, Wang et al. 2011). They observed that the G allele carriers are prone to develop TB in the Tibetan but not Han individuals. This suggests that the miR-146a rs2910164 variants play different roles in the two populations.

There are limited studies focusing on rs2910164 and the role of miR-146a in CVD. Expression of miR-146a was upregulated in the PBMCs of patients with acute coronary syndrome (ACS), including stable angina and AMI. The overexpression of miR-146a correlated with increased expression of proinflammatory cytokines, a critical factor in atherosclerosis (Jamaluddin, Weakly et al. 2011).

In a more recent study recent study investigating the possible relationship between rs2910164 and ACS in a Chinese population found that the GC genotype was significantly associated with decreased risk of ACS. Further analysis showed that the decreased risk was predominant in male subjects with body mass index more than 24 kg/m², and in hypertensive subjects (Huang, Lv et al. 2015).

Xiong *et al.* evaluated the influence of rs2910164 on individual risk for CAD in a Chinese population. The CC genotype was associated with increased susceptibility of developing CAD compared to individuals with the GG and GC genotypes. This increased risk was speculated to be due to the influence of the polymorphism on miR-146a expression levels. Individuals carrying the C allele exhibited a higher expression of mature miR-146a (Xiong, Cho et al. 2014).

The Tampere Vascular Study found an increase in miR-146a expression in atherosclerotic arteries compared to control arteries (Raitoharju, Lyytikainen et al. 2011). This highlights the potential of miR-146a as a biomarker for cardiac events.

References

- Achari, V. and A. K. Thakur (2004). "Association of Major Modifiable Risk Factors Among Patients with Coronary Artery Disease - A Retrospective Analysis." The Journal of the Association of Physicians of India **52**: 103-108.
- Aggarwal, A., S. Aggarwal, et al. (2012). "A retrospective case-control study of modifiable risk factors and cutaneous markers in Indian patients with young coronary artery disease." Journal of the Royal Society of Medicine Cardiovascular Disease **1**(8): 1-8.
- Akkız, H., S. Bayram, et al. (2011). "Genetic Variation in the MicroRNA-499 Gene and Hepatocellular Carcinoma Risk in a Turkish Population: Lack of Any Association in a Case-Control Study." Asian Pacific Journal of Cancer Prevention **12**: 3107-3112.
- Al-Lawati, J. A., A. J. Mohammed, et al. (2003). "Prevalence of the Metabolic Syndrome Among Omani Adults." Diabetes Care **26**(6): 1781-1785.
- Alberti, K. G. M. M., R. H. Eckel, et al. (2009). "Harmonizing the Metabolic Syndrome: A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity." Circulation **120**(16): 1640-1645.
- Ambrose, J. A. and R. S. Barua (2004). "The pathophysiology of cigarette smoking and cardiovascular disease: An update." Journal of the American College of Cardiology **43**(10): 1731-1737.
- Ardekani, A. M. and M. M. Naeini (2010). "The Role of MicroRNAs in Human Diseases." Avicenna Journal of Medical Biotechnology **2**(4): 161-179.
- Arici, M. and J. Walls (2001). "End-stage renal disease, atherosclerosis, and cardiovascular mortality: is C-reactive protein the missing link?" Kidney Int **59**(2): 407-414.
- Bampali, K., K. Mouzarou, et al. (2014). "Genetics and Coronary Artery Disease: Present and Future." Hellenic Journal of Cardiology **55**: 156-163.
- Bartel, D. P. (2004). "MicroRNAs: Genomics, Biogenesis, Mechanism, and Function." Cell **116**(2): 281-297.
- Carmel, R. and D. W. Jacobsen (2001). Homocysteine in Health and Disease, Cambridge University Press.
- Catucci, I., R. Yang, et al. (2010). "Evaluation of SNPs in miR-146a, miR196a2 and miR-499 as low-penetrance alleles in German and Italian familial breast cancer cases." Human Mutation **31**(1): 1052-1057.
- Charchar, F. J., L. D. S. Bloomer, et al. (2012). "Inheritance of coronary artery disease in men: an analysis of the role of the Y chromosome." The Lancet **379**(9819): 915-922.

- Chatzikyriakidou, A., P. V. Voulgari, et al. (2010). "A polymorphism in the 3'-UTR of interleukin-1 receptor-associated kinase (IRAK1), a target gene of miR-146a, is associated with rheumatoid arthritis susceptibility." Joint Bone Spine **77**(5): 411-413.
- Chen, K., F. Song, et al. (2008). "Polymorphisms in microRNA targets: a gold mine for molecular epidemiology." Carcinogenesis **29**(7): 1306-1311.
- Chilton, R. J. (2004). "Pathophysiology of Coronary Heart Disease: A Brief Review." JAOA: Journal of the American Osteopathic Association **104**(9 suppl): 5S-8S.
- Chung, J. Y., Y. C. Park, et al. (2002). "All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction." Journal of Cell Science **115**(4): 679-688.
- Coffey, M., G. K. Crowder, et al. (2003). "Reducing Coronary Artery Disease by Decreasing Homocysteine Levels." Critical Care Nurse **23**(1): 25-30.
- Contu, R., M. V. Latronico, et al. (2010). "Circulating microRNAs as potential biomarkers of coronary artery disease: a promise to be fulfilled?" Circulation Research **107**: 573-574.
- Creemers, E. E., A. J. Tijssen, et al. (2010). "Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease?" Circulation Research **110**(93): 1-17.
- Crowther, M. A. (2005). "Pathogenesis of Atherosclerosis." ASH Education Program Book(1): 436-441.
- Cui, Y., H. Wang, et al. (2012). "Genetic analysis of the SIRT1 gene promoter in myocardial infarction." Biochemical and Biophysical Research Communications **426**(2): 232-236.
- Dahlöf, B. (2010). "Cardiovascular Disease Risk Factors: Epidemiology and Risk Assessment." The American Journal of Cardiology **105**(1, Supplement): 3A-9A.
- Dai, R. and S. A. Ahmed (2011). "MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases." Translational Research **157**(4): 163-179.
- Danaraj, T. J., M. S. Acker, et al. (1959). "Ethnic group differences in coronary heart disease in Singapore: An analysis of necropsy records." American Heart Journal **58**(4): 516-526.
- Das, S. K. and S. C. Elbein (2006). "The genetic basis of type 2 diabetes." Cellscience **2**: 581-589.
- Davis, N. E. (2005). "Atherosclerosis - An Inflammatory Process." JOURNAL OF INSURANCE MEDICINE **37**: 72-75.
- Debrececi, B. and L. Debrececi (2012). "Why Do Homocysteine-Lowering B Vitamin and Antioxidant E Vitamin Supplementations Appear To Be Ineffective in the Prevention of Cardiovascular Diseases?" Cardiovascular Therapeutics **30**(4): 227-233.
- Dzimiri, N., J. J. Wang, et al. (2012). Coronary Artery Disease - New Insights and Novel Approaches. A. Squeri. Janeza Trdine 9, 51000 Rijeka, Croatia, InTech.
- Dzimiri, N., J. J. Wang, et al. (2012). CORONARY ARTERY DISEASE – NEW INSIGHTS AND NOVEL APPROACHES. A. Squeri. Janeza Trdine 9, 51000 Rijeka, Croatia, InTech: 1-270.

- El-Shal, A. S., N. M. Aly, et al. (2013). "Association of microRNAs genes polymorphisms with rheumatoid arthritis in Egyptian female patients." Joint Bone Spine **80**: 626-631.
- Enas A Enas, Annamalai Senthilkumar, et al. (2005). "Dyslipidaemia among Indo-Asians strategies for identification and management." The British Journal of Diabetes & Vascular Disease **5**(81): 81-90.
- Enas, E. A., M. V, et al. (2007). "The Metabolic Syndrome and Dyslipidemia Among Asian Indians: A Population With High Rates of Diabetes and Premature Coronary Artery Disease." Journal of Cardiometabolic Syndrome **2**(4): 267-275.
- Fichtlscherer, S., S. De Rosa, et al. (2010). "Circulating MicroRNAs in Patients With Coronary Artery Disease." Circulation Research **107**(5): 677-684.
- Finkel, T., C.-X. Deng, et al. (2009). "Recent progress in the biology and physiology of sirtuins." Nature **460**(7255): 587-591.
- Flannery, S. and A. G. Bowie (2010). "The interleukin-1 receptor-associated kinases: Critical regulators of innate immune signalling." Biochemical Pharmacology **80**(12): 1981-1991.
- Fung, T. T., E. B. Rimm, et al. (2001). "Association between dietary patterns and plasma biomarkers of obesity and cardiovascular disease risk." The American Journal of Clinical Nutrition **73**(1): 61-67.
- Fuster V and K. BB (2010). Institute of Medicine (US) Committee on Preventing the Global Epidemic of Cardiovascular Disease: Meeting the Challenges in Developing Countries. Promoting Cardiovascular Health in the Developing World: A Critical Challenge to Achieve Global Health. Washington (DC), National Academies Press (US). **2**.
- Galkina, E. and K. Ley (2009). "Immune and inflammatory mechanisms of atherosclerosis (*)." Annual Review of Immunology **27**: 165-197.
- George, G. P., R. Gangwar, et al. (2011). "Genetic variation in microRNA genes and prostate cancer risk in North Indian population." Molecular Biology Reports **38**: 1609-1615.
- Girija, G. (1997). Cardiovascular Disease Prevention - American Heart Association Heart and Stroke Statistical Update. **26**: 78-83.
- Goldring, J. P. D. (2012). "Protein Quantification Methods to Determine Protein Concentration Prior to Electrophoresis." Methods in Molecular Biology **869**: 29-35.
- Gottipati, S., N. L. Rao, et al. (2008). "IRAK1: A critical signaling mediator of innate immunity." Cell Signalling **20**: 269-276.
- Grundey, S. M. (2008). "Metabolic Syndrome Pandemic." Arteriosclerosis, Thrombosis, and Vascular Biology **28**(629-636).
- Hage, F. G. and A. J. Szalai (2007). "C-Reactive Protein Gene Polymorphisms, C-Reactive Protein Blood Levels, and Cardiovascular Disease Risk." Journal of the American College of Cardiology **50**(12): 1115-1122.

- Handy, D. E., R. Castro, et al. (2011). "Epigenetic Modifications : Basic Mechanisms and Role in Cardiovascular Disease." Circulation **123**: 2145-2156.
- Hansson, G. K. (2005). "Inflammation, Atherosclerosis, and Coronary Artery Disease." New England Journal of Medicine **352**(16): 1685-1695.
- Hansson, G. K., A.-K. L. Robertson, et al. (2006). "INFLAMMATION AND ATHEROSCLEROSIS." Annual Review of Pathology: Mechanisms of Disease **1**(1): 297-329.
- Hashemi, M., E. Eskandari-Nasab, et al. (2013). "Association of pre-miRNA-146a rs2910164 and pre-miRNA-499 rs3746444 polymorphisms and susceptibility to rheumatoid arthritis." Molecular Medicine Reports **7**(1): 287-291.
- Horio, Y., T. Hayashi, et al. (2011). "Cellular and molecular effects of sirtuins in health and disease." Clinical Science **121**(5): 191-203.
- Houtkooper, R. H., E. Pirinen, et al. (2012). "Sirtuins as regulators of metabolism and healthspan." Nat Rev Mol Cell Biol **13**(4): 225-238.
- Huang, S., Z. Lv, et al. (2015). "A Genetic Variant in Pre-miR-146a (rs2910164 C>G) Is Associated with the Decreased Risk of Acute Coronary Syndrome in a Chinese Population." The Tohoku journal of experimental medicine **237**(3): 227-233.
- Hughes, K., K. C. Lun, et al. (1990). "Cardiovascular diseases in Chinese, Malays, and Indians in Singapore. I. Differences in mortality." Journal of Epidemiology and Community Health **44**(1): 24-28.
- Hung, P. S., K. W. Chang, et al. (2012). "Association between the rs2910164 polymorphism in pre-miR-146a and oral carcinoma progression." Oral Oncology **48**: 404-408.
- Ignarro, L. J., M. L. Balestrieri, et al. (2007). Nutrition, physical activity, and cardiovascular disease: An update. Cardiovascular Research **73**(2): 326-340.
- Jamaluddin, M. S., S. M. Weakly, et al. (2011). "miRNAs: roles and clinical applications in vascular disease." Expert Rev Mol Diagn **11**(1): 79-89.
- Janssens, S. and R. Beyaert (2003). "Functional Diversity and Regulation of Different Interleukin-1 Receptor-Associated Kinase (IRAK) Family Members." Molecular Cell **11**: 293-302.
- Jazdzewski, K., S. Liyanarachchi, et al. (2009). "Polymorphic mature microRNAs from passenger strand of pre-miR-146a contribute to thyroid cancer." Proceedings of the National Academy of Sciences **106**(5): 1502-1505.
- Jazdzewski, K., E. L. Murray, et al. (2008). "Common SNP in pre-miR-146a decreases mature miR expression and predisposes to papillary thyroid carcinoma." Proceedings of the National Academy of Sciences **105**(20): 7269-7274.
- Jin, Y. and C. Lee (2013). "Single Nucleotide Polymorphisms Associated with MicroRNA Regulation." Biomolecules **3**(2): 287.

- Jousilahti, P., E. Vartiainen, et al. (1999). "Sex, Age, Cardiovascular Risk Factors, and Coronary Heart Disease: A Prospective Follow-Up Study of 14 786 Middle-Aged Men and Women in Finland." Circulation **99**(9): 1165-1172.
- Kaiser, J. (2002). "Biobanks: population databases boom, from Iceland to the US." Science **298**: 1158-1161.
- Kaur, J. (2014). "A Comprehensive Review on Metabolic Syndrome." Cardiology Research and Practice **2014**: 21.
- Kearney, P. M., M. Whelton, et al. (2005). "Global burden of hypertension: analysis of worldwide data." Lancet **365**(9455): 217-223.
- Klein, L. W. and S. Nathan (2003). "Coronary artery disease in young adults*." Journal of the American College of Cardiology **41**(4): 529-531.
- Kluijtmans, L. A. J. and A. S. Whitehead (2001). "Methylenetetrahydrofolate reductase genotypes and predisposition to atherothrombotic disease." European Heart Journal **22**: 294-299.
- Kozomara, A. and S. Griffiths-Jones (2014). "miRBase: annotating high confidence microRNAs using deep sequencing data." Nucleic Acids Research **42**(Database issue): D68-D73.
- Kozomara, A. and S. Griffiths-Jones (2014). "miRBase: annotating high confidence microRNAs using deep sequencing data." Nucleic Acids Research **42**(Database issue): 68-73.
- Kullo, I. J. and K. Ding (2007). "Mechanisms of Disease: the genetic basis of coronary heart disease." Nature Clinical Practice Cardiovascular Medicine **4**(10): 558-569.
- Lal, S. (2004). Genetic epidemiology of coronary artery disease. Master of Science, National University of Singapore.
- Lanktree, M. (2008). "Genetic Basis of Coronary Artery Disease." University Of Western Ontario Medical Journal **77**(2): 55-58.
- Lavie, C. J., R. V. Milani, et al. (2009). "Obesity and Cardiovascular Disease: Risk Factor, Paradox, and Impact of Weight Loss." Journal of the American College of Cardiology **53**(21): 1925-1932.
- Lee, R. C., R. L. Feinbaum, et al. (1993). "The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*." Cell **3**(75): 843-854.
- Levenson, J. W., P. J. Skerrett, et al. (2002). "Reducing the Global Burden of Cardiovascular Disease: The Role of Risk Factors." Preventive Cardiology **5**(4): 188-199.
- Li, D., T. Wang, et al. (2011). "Genetic study of two single nucleotide polymorphisms within corresponding microRNAs and susceptibility to tuberculosis in a Chinese Tibetan and Han population." Human Immunology **72**(7): 598-602.
- Li, D., T. Wang, et al. (2011). "Genetic study of two single nucleotide polymorphisms within corresponding microRNAs and susceptibility to tuberculosis in a Chinese Tibetan and Han population." Human Immunology **72**: 598-602.

- Li, J. J. and J. L. Chen (2005). "Inflammation may be a bridge connecting hypertension and atherosclerosis." Medical Hypotheses **64**(5): 925-929.
- Li, L.-J., L.-B. Gao, et al. (2011). "Association between SNPs in pre-miRNA and risk of chronic obstructive pulmonary disease." Clinical Biochemistry **44**(10–11): 813-816.
- Li, L., X. Chen, et al. (2010). "MicroRNA-146a and human disease." Scandinavian Journal of Immunology **71**: 227-231.
- Libby, P., P. M. Ridker, et al. (2002). "Inflammation and Atherosclerosis." Circulation **105**(9): 1135-1143.
- Libby, P. and P. Theroux (2005). "Pathophysiology of Coronary Artery Disease." Circulation **111**(25): 3481-3488.
- Lin, Y. and Z. Sun (2010). "Current reviews on type 2 diabetes." Journal of Endocrinology **204**: 1-11.
- Ling, C. and L. Groop (2009). "Epigenetics: A Molecular Link Between Environmental Factors and Type 2 Diabetes." Diabetes **58**: 2718-2725.
- Liu, X., K. Fortin, et al. (2008). "MicroRNAs: Biogenesis and Molecular Functions." Brain Pathology **18**(1): 113-121.
- Lowery, A. J., N. Miller, et al. (2008). "MicroRNAs as Prognostic Indicators and Therapeutic Targets: Potential Effect on Breast Cancer Management." Clinical Cancer Research **14**(2): 360-365.
- Mack, C. P. (2008). "An Epigenetic Clue to Diabetic Vascular Disease." Circulation Research **103**: 568-570.
- Malaguarnera, M., M. Vacante, et al. (2013). "Lipoprotein(a) in Cardiovascular Diseases." BioMed Research International **2013**: 9.
- Mearns, B. M. (2012). "Coronary artery disease: Y chromosome link to CAD risk." Nat Rev Cardiol **9**(4): 187-187.
- Mehlig, K., K. Leander, et al. (2013). "The association between plasma homocysteine and coronary heart disease is modified by the MTHFR 677C>T polymorphism." Heart.
- Menghini, R., R. Stöhr, et al. (2014). "MicroRNAs in vascular aging and atherosclerosis." Ageing Research Reviews **17**: 68-78.
- Misra, A., J. S. Wasir, et al. (2005). "An Evaluation of candidate definitions of the Metabolic syndrome in adult Asian Indians." Diabetes Care **28**: 398-403.
- Mittal, R. D., R. Gangwar, et al. (2011). "Investigative role of pre-microRNAs in bladder cancer patients: a case-control study in North India." DNA AND CELL BIOLOGY **30**(6): 401-406.
- Mohan, V., S. Sandeep, et al. (2007). "A diabetes risk score helps identify metabolic syndrome and cardiovascular risk in Indians - the Chennai Urban Rural Epidemiology Study (CURES-38)." Diabetes, obesity & metabolism **9**(3): 337-343.
- Moore, L. D., T. Le, et al. (2013). "DNA Methylation and Its Basic Function." Neuropsychopharmacology **38**(1): 23-38.

- Muroi, M. and K.-i. Tanamoto (2012). "IRAK-1-mediated negative regulation of Toll-like receptor signaling through proteasome-dependent downregulation of TRAF6." Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1823**(2): 255-263.
- Murray, C. J. and A. D. Lopez (1997). "Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study." The Lancet **349**: 1498-1504.
- Narverud, I., K. Retterstol, et al. (2014). "Markers of atherosclerotic development in children with familial hypercholesterolemia: A literature review." Atherosclerosis **235**(2): 299-309.
- Nasir, K., E. D. Michos, et al. (2004). "Coronary Artery Calcification and Family History of Premature Coronary Heart Disease: Sibling History Is More Strongly Associated Than Parental History." Circulation **110**(15): 2150-2156.
- Nazari-Jahantigh, M., V. Egea, et al. (2014). "MicroRNA-specific regulatory mechanisms in atherosclerosis." Journal of Molecular and Cellular Cardiology **89**: 35-41.
- NHLBI. (2013). "Health Information for the Public - Coronary Artery Disease." Retrieved 5 October, 2015, from <http://www.nhlbi.nih.gov/health/health-topics/topics/heartattack#>.
- Norman, R. B. D., M. Schneider, et al. (2006). Revised Burden of Disease Estimates for the comparative risk factor assessment, South Africa 2000. Cape Town: Medical Research Council 2006.
- O'Neill, L. A. J. (2008). "The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress." Immunological Reviews **226**(1): 10-18.
- Okraïec, K., D. K. Banerjee, et al. (2004). "Coronary artery disease in the developing world." American Heart Journal **148**(1): 7-15.
- Olafiranye, O., F. Zizi, et al. (2011). "Management of Hypertension among Patients with Coronary Heart Disease." International Journal of Hypertension **2011**: 6.
- Olokoba, A. B., O. A. Obateru, et al. (2012). "Type 2 Diabetes Mellitus: A Review of Current Trends." Oman Medical Journal **27**(4): 269-273.
- Omae, T., T. Nagaoka, et al. (2013). "Homocysteine Inhibition of Endothelium-Dependent Nitric Oxide-Mediated Dilation of Porcine Retinal Arterioles via Enhanced Superoxide Production Homocysteine-Induced Retinal Vascular Dysfunction." Investigative Ophthalmology & Visual Science **54**(3): 2288-2295.
- Parikh, R. M. and V. Mohan (2012). "Changing definitions of metabolic syndrome." Indian Journal of Endocrinology and Metabolism **16**(1): 7-12.
- Park, Y.-W., S. Zhu, et al. (2003). "The Metabolic Syndrome: Prevalence and Associated Risk Factor Findings in the US Population From the Third National Health and Nutrition Examination Survey, 1988-1994." Archives of internal medicine **163**(4): 427-436.
- Pauley, K. M., M. Satoh, et al. (2008). "Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients." Arthritis Research & Therapy **10**(4).

- Perry, M. M., S. A. Moschos, et al. (2008). "Rapid Changes in MicroRNA-146a Expression Negatively Regulate the IL-1 β -Induced Inflammatory Response in Human Lung Alveolar Epithelial Cells." The Journal of Immunology **180**(8): 5689-5698.
- Poirier, P., T. D. Giles, et al. (2006). "Obesity and Cardiovascular Disease: Pathophysiology, Evaluation, and Effect of Weight Loss: An Update of the 1997 American Heart Association Scientific Statement on Obesity and Heart Disease From the Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism." Circulation **113**(6): 898-918.
- Pons, D., F. R. de Vries, et al. (2009). "Epigenetic histone acetylation modifiers in vascular remodelling: new targets for therapy in cardiovascular disease." European Heart Journal **30**: 266-277.
- Raitoharju, E., L. P. Lyytikainen, et al. (2011). "miR-21, miR-210, miR-34a, and miR-146a/b are up-regulated in human atherosclerotic plaques in the Tampere Vascular Study." Atherosclerosis **219**: 211-217.
- Rajadurai, J., J. Arokiasamy, et al. (1992). "Coronary artery disease in Asians." Australian and New Zealand Journal of Medicine **22**(4): 345-348.
- Rajeshwari, R., T. A. Nicklas, et al. (2005). "Cardiovascular diseases—a major health risk in Asian Indians." Nutrition research (New York, N.Y.) **25**(6): 515-533.
- Rampal, S., S. Mahadeva, et al. (2012). "Ethnic Differences in the Prevalence of Metabolic Syndrome: Results from a Multi-Ethnic Population-Based Survey in Malaysia." PLoS ONE **7**(9): e46365.
- Ranjith, N., R. J. Pegoraro, et al. (2005). "Demographic data and outcome of acute coronary syndrome in the South African Asian Indian population." Cardiovascular Journal of South Africa **16**(1): 48-54.
- Raupach, T., K. Schafer, et al. (2006). "Secondhand smoke as an acute threat for the cardiovascular system: a change in paradigm." European Heart Journal **27**(4): 386-392.
- Risch, N. J. (2000). "Searching for genetic determinants in the new millennium." Nature **405**: 847-856.
- Rissam, H. S., S. Banzal, et al. Profile of acute myocardial infarction in Indians in Kingdom of Saudi Arabia (unpublished data).
- Rissam, H. S., S. Kishore, et al. (2001). "Coronary Artery Disease in Young Indians - The Missing Link." Journal, Indian Academy of Clinical Medicine **2**(3):128-132.
- Roberts, R. and A. F. R. Stewart (2012). "Genes and Coronary Artery Disease - Where Are We?" Journal of the American College of Cardiology **60**(18): 1715-1721.
- Roldán, V., A. B. Arroyo, et al. (2014). "Prognostic role of MIR146A polymorphisms for cardiovascular events in atrial fibrillation." Thrombosis and Haemostasis **112**(10): 781-788.
- Rosendorff, C., H. R. Black, et al. (2007). "REPRINT Treatment of Hypertension in the Prevention and Management of Ischemic Heart Disease: A Scientific Statement From the American

- Heart Association Council for High Blood Pressure Research and the Councils on Clinical Cardiology and Epidemiology and Prevention." Hypertension **50**(2): e28-e55.
- Rosendorff, C., D. T. Lackland, et al. (2015). "Treatment of Hypertension in Patients With Coronary Artery Disease A Scientific Statement from the American Heart Association, American College of Cardiology, and American Society of Hypertension." Journal of the American College of Cardiology **65**(18): 1998-2038.
- Rusca, N. and S. Monticelli (2011). "MiR-146a in Immunity and Disease." Molecular Biology International **2011**: 1-7.
- Sadeghi, R., N. Adnani, et al. (2013). "Premature coronary heart disease and traditional risk factors- can we do better?" International Cardiovascular Research Journal **7**(2): 46-50.
- Savoia, C. and E. L. Schiffrin (2007). "Vascular inflammation in hypertension and diabetes: molecular mechanisms and therapeutic interventions." Clinical Science **112**(7): 375-384.
- Sayols-Baixeras, S., C. Lluís-Ganella, et al. (2014). "Pathogenesis of coronary artery disease: focus on genetic risk factors and identification of genetic variants." The Application of Clinical Genetics **7**: 15-32.
- Scanlon P.J., Faxon D.P., et al. (1999). "ACC/AHA guidelines for coronary angiography. A report of the American College of Cardiology/American Heart Association Task Force on practice guidelines (Committee on Coronary Angiography). Developed in collaboration with the Society for Cardiac Angiography and Interventions." Journal of the American College of Cardiology **33**(6): 1756-1824.
- Schächinger, V., M. B. Britten, et al. (1999). "A Positive Family History of Premature Coronary Artery Disease Is Associated With Impaired Endothelium-Dependent Coronary Blood Flow Regulation." Circulation **100**(14): 1502-1508.
- Schnabel, R. B., A. Baccarelli, et al. (2012). "Next Steps in Cardiovascular Disease Genomic Research - Sequencing, Epigenetics, and Transcriptomics." Clinical Chemistry **58**(1): 113-126.
- Schulte, C. and T. Zeller (2015). "microRNA-based diagnostics and therapy in cardiovascular disease - Summing up the facts." Cardiovascular Diagnosis and Therapy **5**(1): 17-36.
- Seedat, Y. K. (2005). "Review: Diabetes mellitus in South African Indians." The British Journal of Diabetes & Vascular Disease **5**(5): 249-251.
- Sesso, H. D., I.-M. Lee, et al. (2001). "Maternal and Paternal History of Myocardial Infarction and Risk of Cardiovascular Disease in Men and Women." Circulation **104**(4): 393-398.
- Shah, T., S. S. Jonnalagadda, et al. (2005). "Prevalence of metabolic syndrome risk factors among young adult Asian Indians." Journal of Immigrant Health **7**(2): 117-126.
- Sharma M, G. M. (2005). "Premature coronary artery disease in Indians and its associated risk factors." Vascular Health and Risk Management **1**(3): 217-225.

- Sharma, S. N., U. Kaul, et al. (1990). "Coronary arteriographic profile in young and old Indian patients with ischaemic heart disease: a comparative study." Indian Heart Journal **42**(5): 365-369.
- Shen, J., C. B. Ambrosone, et al. (2008). "A functional polymorphism in the miR-146a gene and age of familial breast/ovarian cancer diagnosis." Carcinogenesis **29**(10): 1963-1966.
- Shiwaku, K., A. Nogi, et al. (2005). "Prevalence of the Metabolic Syndrome using the Modified ATP III Definitions for Workers in Japan, Korea and Mongolia." Journal of Occupational Health **47**(2): 126-135.
- Stefano, V. D., G. Zaccagnini, et al. (2011). "microRNAs as peripheral blood markers of cardiovascular disease." Vascular Pharmacology **55**: 111-118.
- Stern, L. L., J. B. Mason, et al. (2000). "Genomic DNA Hypomethylation, a Characteristic of Most Cancers, Is Present in Peripheral Leukocytes of Individuals Who Are Homozygous for the C677T Polymorphism in the Methylenetetrahydrofolate Reductase Gene." Cancer Epidemiology, Biomarkers and Prevention **9**: 849-853.
- Stühlinger, M. C., P. S. Tsao, et al. (2001). "Homocysteine Impairs the Nitric Oxide Synthase Pathway: Role of Asymmetric Dimethylarginine." Circulation **104**(21): 2569-2575.
- Sun, H., Q. Li, et al. (2014). "Quantitative assessment of the association between microRNA-499 rs3746444 A/G polymorphism and cancer risk." Tumor Biology **35**: 2351-2358.
- Takeda, K. and S. Akira (2004). "TLR signalling pathways." Seminars in Immunology **16**: 3-9.
- Tan, C.-E., S. Ma, et al. (2004). "Can We Apply the National Cholesterol Education Program Adult Treatment Panel Definition of the Metabolic Syndrome to Asians?" Diabetes Care **27**(5): 1182-1186.
- Tang, Y., X. Luo, et al. (2009). "MicroRNA-146a contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins." Arthritis & Rheumatism **60**(4): 1065-1075.
- Teo, K. K., S. Ounpuu, et al. (2006). "Tobacco use and the risk of myocardial infarction in 52 countries in the INTERHEART study: a case-control study." Lancet **368**(9563): 647-658.
- The Emerging Risk Factors, C. (2010). "C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis." Lancet **375**(9709): 132-140.
- Thompson, P. D., D. Buchner, et al. (2003). "Exercise and Physical Activity in the Prevention and Treatment of Atherosclerotic Cardiovascular Disease: A Statement From the Council on Clinical Cardiology (Subcommittee on Exercise, Rehabilitation, and Prevention) and the Council on Nutrition, Physical Activity, and Metabolism (Subcommittee on Physical Activity)." Circulation **107**(24): 3109-3116.
- van Eijk, K. R., S. de Jong, et al. (2012). "Genetic analysis of DNA methylation and gene expression levels in whole blood of healthy human subjects." BMC Genomics **13**(1): 1-13.

- van Rooij, E. (2011). "The Art of MicroRNA Research." Circulation Research **108**: 219-234.
- Vasisht, S., R. Gulati, et al. (2002). "Polymorphism (C677T) in the 5,10-Methylenetetrahydrofolate reductase (MTHFR) Gene: A Preliminary Study in North Indian Men." Indian Journal of Clinical Biochemistry **17**(1): 99-107.
- Virdis, A. and E. L. Schiffrin (2003). "Vascular inflammation: a role in vascular disease in hypertension?" Current Opinion in Nephrology and Hypertension **12**(2): 181-187.
- Wainwright, J. (1966). "Cardiovascular disease in the Asiatic (Indian) population of Durban." South African medical journal **43**(6): 136-138.
- Webster, A. L. H., M. S. C. Yan, et al. (2013). "Epigenetics and Cardiovascular Disease." Canadian Journal of Cardiology **29**: 46-57.
- Wild, S. and P. McKeigue (1997). "Cross sectional analysis of mortality by country of birth in england and wales, 1970-92." BMJ **314**(7082): 705.
- Wilkinson, P., J. Sayer, et al. (1996). "Comparison of case fatality in south Asian and white patients after acute myocardial infarction: observational study." BMJ : British Medical Journal **312**(7042): 1330-1333.
- Willett, W. C., A. Green, et al. (1987). "Relative and absolute risks of coronary heart disease among women who smoke cigarettes." New England Journal of Medicine **317**: 1303-1309.
- Wilson, P. W. (2004). "Assessing coronary heart disease risk with traditional and novel risk factors." Clinical Cardiology **6**(3).
- Wright, M. W. and E. A. Bruford (2011). "Naming 'junk': Human non-protein coding RNA (ncRNA) gene nomenclature." Human Genomics **5**(2): 90-98.
- Xiong, X.-d., M. Cho, et al. (2014). "A common variant in pre-miR-146 is associated with coronary artery disease risk and its mature miRNA expression." Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis **761**: 15-20.
- Xiong, X. D., M. Cho, et al. (2014). "A common variant in pre-miR-146 is associated with coronary artery disease risk and its mature miRNA expression." Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis(1386-1964 (Print)).
- Xu, B., N. H. Feng, et al. (2010). "A functional polymorphism in Pre-miR-146a gene is associated with prostate cancer risk and mature miR-146a expression in vivo." The Prostate **70**: 467-472.
- Xu, T., Y. Zhu, et al. (2008). "A functional polymorphism in the miR-146a gene is associated with the risk for hepatocellular carcinoma." Carcinogenesis **29**(11): 2126-2131.
- Yang, B., J. Chen, et al. (2012). "Association of polymorphisms in pre-miRNA with inflammatory biomarkers in rheumatoid arthritis in the Chinese Han population." Human Immunology **73**(1): 101-106.
- Yue, C., M. Wang, et al. (2011). "Polymorphism of the pre-miR-146a is associated with risk of cervical cancer in a Chinese population." Gyneologic Oncology **122**: 33-37.

- Zhang, J., B. Yang, et al. (2011). "Association of pre-microRNAs genetic variants with susceptibility in systemic lupus erythematosus." Molecular Biology Reports **38**: 1463-1468.
- Zhi, H., L. Wang, et al. (2012). "Polymorphisms of miRNAs genes are associated with the risk and prognosis of coronary artery disease." Clinical Research in Cardiology **101**: 289-296.
- Zilahi, E., T. Tarr, et al. (2011). "Increased microRNA-146a/b, TRAF6 gene and decreased IRAK1 gene expressions in the peripheral mononuclear cells of patients with Sjögren's syndrome." Immunology Letters **141**: 165-168.

CHAPTER 3

Methylenetetrahydrofolate reductase C677T polymorphism is associated with increased risk of coronary artery disease in young South African Indians

Prithiksha Ramkaran^a, Alisa Phulukdaree^b, Sajidah Khan^c, Devapregasan Moodley^a, Anil A. Chuturgoon^{a*}

^aDiscipline of Medical Biochemistry and Chemical Pathology, Howard College Campus, University of KwaZulu-Natal, Durban, South Africa

^bDepartment of Physiology, School of Medicine, Faculty of Health Sciences, Prinshof Campus, University of Pretoria, South Africa

^cDepartment of Cardiology, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa

*Corresponding author : School of Laboratory Medicine and Medical Sciences, Discipline of Medical Biochemistry and Chemical Pathology, George Campbell Building - South Entrance, 3rd Floor, Kind George V Avenue, Howard College Campus, University of KwaZulu-Natal, Durban, South Africa. E-mail address: CHUTUR@ukzn.ac.za (A.A. Chuturgoon).

Gene (2015), 571(1), 28-32

[DOI: 10.1016/j.gene.2015.06.044](https://doi.org/10.1016/j.gene.2015.06.044)

ABSTRACT

Methylenetetrahydrofolate reductase (MTHFR) reduces 5',10'-methylenetetrahydrofolate to 5'-methyltetrahydrofolate, and is involved in remethylation of homocysteine to methionine, two important reactions involved in folate metabolism and methylation pathways. The common MTHFR C677T single nucleotide polymorphism (SNP) (rs1801133) has been associated with raised levels of homocysteine, a well known risk factor for coronary artery disease (CAD). CAD is a major cause of mortality worldwide. The age of onset of this chronic disorder is on the decline, particularly in the Indian population. Indians in South Africa (SA) have a higher prevalence of premature CAD compared to Black South Africans. The MTHFR C677T SNP has not been investigated in the SA Indian population. The present study therefore investigated the MTHFR C677T SNP in young SA Indian males with CAD compared to young Indian and Black male controls. A total of 290 subjects were recruited into this study which included 106 CAD patients (diagnosed on angiography, mean age 37.5, range 24–45 years), 100 Indian male controls (mean age 37.5, range 28–45 years), and 84 Black male controls (mean age 36.4, range 25–45). Polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) was used to genotype CAD patients and healthy controls. Data for clinical markers were obtained from pathology reports. There was a significant association between the 677MTHFR variant (T) allele and CAD patients compared to the healthy Indian controls ($p=0.0353$, OR=2.105 95% CI 1.077–4.114). Indian controls presented with a higher frequency of the variant allele compared to Black controls (7% vs. 2% respectively, $p = 0.0515$ OR = 3.086 95% CI 0.9958–9.564). The MTHFR C677T SNP did not influence levels of total cholesterol, LDL, HDL, triglycerides, fasting glucose, fasting insulin, HbA1c or hsCRP. The higher frequency of the MTHFR 677 variant allele in South African Indians may be a contributing factor to the higher risk profile for the development of premature CAD in Indians.

Key Words

Methylenetetrahydrofolate reductase; MTHFR C677T; Single-nucleotide polymorphism; Homocysteine; Premature coronary artery disease

1. Introduction

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme that catalyses the reduction of 5',10'-methylene tetrahydrofolate to 5'-methyltetrahydrofolate; a major form of folate in plasma, and a carbon donor for the remethylation of homocysteine to methionine (Kluijtmans and Whitehead 2001; Vasisht, Gulati et al. 2002). The enzyme is therefore responsible for reducing levels of homocysteine in the plasma.

The 2.2kb long MTHFR gene is located on the short arm of chromosome 1 at 1p36.3. A common SNP at position 677 of the MTHFR gene (rs1801133) in which a cytosine is converted to thymine, results in the substitution of an alanine residue to valine in the enzyme (Anderson, King et al. 1997). The encoded protein has reduced activity at 37°C and higher, and thus the C677T SNP is termed “thermolabile” (Vasisht, Gulati et al. 2002). The MTHFR 677 TT genotype has been linked to increased plasma homocysteine levels (Anderson, King et al. 1997; Clarke, Bennett et al. 2012; Mehlig, Leander et al. 2013; Nienaber-Rousseau, Ellis et al. 2013).

Elevated total plasma homocysteine (tHcy) is an established risk factor for CAD that appears to occur independently of other conventional risk factors (Doshi, McDowell et al. 2002). There are several proposed mechanisms to explain the association between homocysteine and CAD, including endothelial cell dysfunction, enhanced platelet aggregation, production of free radicals, and stimulation of oxidation of low-density lipoprotein (LDL) (Stanger, Semmelrock et al. 2002; Coffey, Crowder et al. 2003).

Dietary intake and status of folate, and to a lesser extent the status of vitamins B2, B6, and B12, are classical determinants of plasma homocysteine (Muskiet 2005). Oral folate supplements can effectively lower plasma homocysteine levels (Clarke 1998). The metabolism of homocysteine and folate are interrelated, and increasing folate intake enhances remethylation of homocysteine. A daily dose of 0.4-0.5 mg of folate is reported to reduce tHcy by 25% (Doshi, McDowell et al. 2002). This has led to the suggestion that folate supplementation may reduce the risk of CAD and other vascular-related disease by reducing tHcy.

Coronary artery disease is a multifactorial disease that depends on the interaction between environmental risk factors and several predisposing genes (Andreassi, Botto et al. 2003). On a genetic basis, SNPs in key genes may play a vital role in an individual's susceptibility and response to the disease.

Although the relationship between the MTHFR C677T variant and risk of CAD is not yet fully understood, the SNP may be important in disease mechanisms underlying CAD due to its influence on tHcy levels. The role of the MTHFR C677T SNP in CAD has been investigated across a range of ethnic groups (Gudnason, Stansbie et al. 1998; Friso, Girelli et al. 2002; Sun, Xu et al. 2005). Indian populations worldwide have the highest prevalence of early-onset CAD compared to other ethnic groups (Rajeshwari, Nicklas et al. 2005). Early-onset or premature CAD is defined as cardiac events occurring before the age of 55 in men and 65 in women, and before the age of 40 in its severe form (Rissam, Kishore et al. 2001).

People of Indian ancestry in particular develop heart disease when they younger than 40, a phenomenon unheard of in other populations (Sastry, Golla et al. 2011). The pattern with regards to early-onset CAD in the Indian population is similar in various parts of the world. In India, 25-40% of patients suffering from acute myocardial infarction (AMI) were below the age of 45 (Girija 1997). In Great Britain, AMI occurred in Indians younger than 40 years old – and the incidence was 10 times higher than the local Caucasian population (Rajadurai, Arokiasamy et al. 1992). Studies in Singapore reported mortality due to CAD below 30 years of age occurs 10 times more in the Indian population compared to the local Chinese (Hughes, Lun et al. 1990). In the Middle East, 80% of patients who experienced AMI were Indians below the age of 40, yet Indians account for approximately 10% of the population in this region (Rissam, Banzal et al. ; Rissam, Kishore et al. 2001). Angiographically, Indians have a 15 times higher rate of CAD than Chinese and 10 times higher rate than Malays below 40 years of age (Rissam, Kishore et al. 2001).

The occurrence of CAD is on the rise in developing countries such as South Africa. Most South African Indians are descendants of indentured labourers who were brought to KwaZulu-Natal from India between 1860 and 1911 (Seedat 2005). Indians in India and South Africa are predisposed to the early onset of CAD, one to two decades earlier than other population groups, indicating a genetic link (Ranjith, Pegoraro et al. 2005; Sharma M 2005). In 1966, Wainwright reported a high incidence of early-onset CAD in South African Indians (Wainwright 1966). In 2006, reports still showed that the highest death rates for early-onset CAD in South Africa occur in Indians, followed by mixed race, White, and Black (Norman, Schneider et al. 2006). This suggests that regardless of the number of generations of Indian immigrants who have lived in South Africa, there remains a differing pattern of early-onset CAD mortality compared to the natives of the country, despite them sharing the same environment and to some extent similar diets and cultural habits (Lal 2004). There is currently no literature available on South African Indians with regard to the MTHFR C677T SNP. We set out to assess this SNP in a cohort of young South African Indians with CAD compared to Indian and Black male controls.

2. Methods

2.1. Patient recruitment and sample collection

A total of 106 young SA Indian male CAD patients (mean age 37.5, range 24–45 years), 100 Indian male controls (mean age 37.5, range 28–45 years), and 84 Black male controls (mean age 36.4, range 25–45) were enrolled following institutional ethical approval (BE067/14). A full pathology report of clinical markers was assessed by routine laboratory testing at Global Clinical and Viral Laboratory (Amanzimtoti, South Africa) — a South African National Accreditation System (SANAS) certified laboratory. The following parameters were tested: Haematology (Roche Sysmex 1800XT), Chemistry (Beckman Coulter DXC600), Endocrinology and hsCRP (Siemens Centaur XP), Serology (BD Biosciences FACS Calibur) as per international standards to obtain levels of total cholesterol, HDL, LDL, triglycerides, fasting glucose, 2 h glucose, fasting insulin, glycosylated haemoglobin, sodium, potassium, bicarbonate, chloride, urea, creatinine, glomerular filtration rate, cluster of differentiation (CD) 4 count, CD8 count, CD45 count and CD3 count. The physical measurements of weight, height, abdominal circumference, waist circumference, and patient history were conducted by the clinician. The inclusion criteria for young CAD patients were: Indian ancestry and unrelated, adults aged \geq 18 years, and stable CAD confirmed on angiography. Exclusion criteria for patients were an acute coronary syndrome/ revascularisation procedure in the preceding three months, chronic renal or liver disease, malignancy and known active inflammatory or infectious disease. Indian and Black male controls who did not have heart disease were recruited. Inclusion criteria for controls were Indian/African ancestry, unrelated to one another, adult males below the age of 45 years and no known or suspected atherosclerotic vascular disease. For control subjects, a blood sample was drawn and in addition an exercise electrocardiogram (ECG) was recorded in order to exclude heart disease. The exclusion criteria for controls were as for cases, and included the following: symptoms or clinical evidence of atherosclerotic vascular disease i.e. CAD, stroke/carotid disease or peripheral vascular disease, evidence of ischaemia/infarction on 12 lead resting ECG or evidence of ischaemia on treadmill exercise stress testing done to predict maximum heart rate. A positive family history was recorded if any first degree relative (father, mother, brother or sister) had CAD (Table 2).

2.2. DNA extraction

Genomic DNA was extracted from the whole blood sample of each patient and control according to the method described by Sambrook et al. 2001 (Sambrook and Russell 2001). Cells were transferred to 600 μ l lysis buffer (0.5% sodium dodecyl sulphate (SDS), 150mM NaCl, 10mM ethylenediaminetetra-acetic acid (EDTA), 10mM Tris-HCl (pH 8.0)). To this, RNase A (100 μ g/mL; DNase-free) was added to the solution and incubated (37°C, 1h). Proteinase K (200 μ g/mL) was then added and incubated (3h, 50°C). Protein contaminants were then precipitated by adding 5mM 0.1% potassium acetate before centrifugation (5000 \times g; 15min). Supernatants containing genomic DNA

were transferred to fresh tubes and extracted with 100% isopropanol on ice and thereafter washed with 70% ethanol. DNA samples were dissolved in 10mM Tris and 0.1mM EDTA (pH 7.4, 4°C). DNA concentration was determined using the NanoDrop 2000 spectrophotometer, and all samples were standardised to a concentration of 10ng/μl.

2.3. Genotyping

A 198 bp amplicon was obtained by polymerase chain reaction (PCR) amplification of the genomic DNA using 40pmol of each primer (forward: 5'-TGAAGGAGAAGGTGTCTGGGGGA-3' and reverse: 5'-AGGACGGTGCGGTGAGAGTG-3'), 200μM of each dNTP, 2.5mM MgCl₂, 1× Green GoTaq Flexi buffer, 0.2U GoTaq DNA polymerase (Promega) and 30ng genomic DNA template. PCR was performed under the following cycling conditions: 95°C for 5min (initial denaturation), followed by 95°C for 1min, 59°C for 1min (annealing), 72°C for 2min, and 72°C for 7min (final extension), for a total of 35 cycles. PCR products were electrophoresed on agarose gel (1.8%, 0.5mg/mL ethidium bromide) and visualised using the Uvitech image documentation system (Uvitech Alliance 2.7).

PCR-RFLP was used to determine the MTHFR C677T genotypes. PCR products were subjected to overnight restriction digestion by *Hinf*I (5 Units) at 37°C. Restriction products were electrophoresed on agarose gel (3%, 0.5mg/mL ethidium bromide) and visualised as was the PCR product. Presence of the wild-type C-allele resulted in no cleavage of the PCR product. Heterozygote C677T yielded three fragments of 198 bp, 175 bp, and 23 bp. Homozygote C677T yielded two fragments of 175 bp and 23 bp (Figure 3.1).

2.4. Statistical analysis

The Hardy–Weinberg equilibrium was used to test for deviation of allele/genotype frequency. All other statistical analysis was performed with Graphpad prism software (version 5.0). Allele and genotype frequencies were calculated using the Fisher's exact and Chi square tests, respectively. Comparisons for clinical characteristics amongst CAD patients, Indian controls, and Black controls were done by performing a one-way ANOVA. Characteristics of CAD patients according to genotype was analysed by a non-parametric T-test. Results are expressed as mean ± standard error. A p value less than 0.05 was considered statistically significant.

3. Results

The MTHFR C677T SNP was investigated using PCR-RFLP. The genotype distribution complied with the Hardy–Weinberg equilibrium in Indian and Black controls (Chi squared $p=0.753$, and $p=0.975$ respectively; Table 3.1).

There was significant association between the 677 MTHFR variant (T) allele and CAD patients compared to the healthy Indian controls ($p=0.0353$, OR =2.105 95% CI 1.077–4.114; Table 3.1). Indian controls presented with a higher frequency of the variant allele compared to Black controls (7% vs. 2% respectively, $p=0.0515$ OR = 3.086 95% CI 0.9958–9.564; Table 3.1).

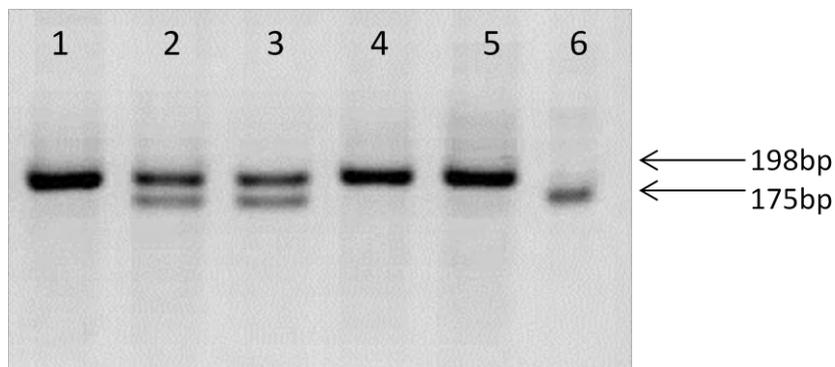


Figure 3.1 *Hinf*I-restricted MTHFR gene fragments. Lane 1: PCR amplicon product, Lane 2 and 3: CT genotype, Lane 4 and 5: CC genotype, Lane 6: TT genotype

Table 3.1 Frequency of MTHFR C677T genotypes and alleles in SA Indian CAD patients and controls (Indian and Black).

	South African Indians		South African Blacks
	CAD Patients (n=106)	Controls (n=100)	Total n=84
Genotypes			
CC n (%)	79 (74.5)	86 (86)	80 (95)
CT n (%)	25 (23.5)	14 (14)	4 (5)
TT n (%)	2 (2)	0 (0)	0 (0)
Alleles			
C n (%)	183 (86)	186 (93)	164 (98)
T n (%)	29 (14)	14 (7)	4 (2)
HWE P value		0.753	0.975
p-value	0.0353 ^a	0.0515 ^b	

Fisher's exact test for heterogeneity between ^a healthy SA Indian and coronary artery disease (CAD) patients and ^b healthy SA Black and healthy SA Indian

HWE: Hardy-Weinberg equilibrium

As expected, CAD patients presented with more conventional risk factors (diabetes, hypertension, smoking, triglycerides, fasting glucose, HBA1c, and hsCRP) than the control groups (Table 3.2).

Table 3.2 Demographics and clinical parameters for CAD patients, Indian, and Black controls

	CAD Patients (n=106)	Indian Controls (n=100)	Black controls (n=84)	p-value
Prediabetic/diabetic n (%)	63 (59)	39 (39)	12 (14)	0.0036 ^a 0.0002 ^b
Hypertensive n (%)	44 (42)	22 (22)	2 (2)	0.0029 ^a p<0.0001 ^b
Positive family history of CAD (%)	85	47	1	p<0.0001 ^a p<0.0001 ^b
Smokers/Ex-smokers	96	65	43	p<0.0001 ^a
Non-smokers	10	35	41	0.0715 ^b
Age (years)	37.6	37.5	36.4	0.9256 ^a 0.1080 ^b
BMI (kg/m ²)	28.15±0.45	26.31±0.55	28.95±0.51	0.0104 ^a 0.6275 ^b
Laboratory parameters				
Total cholesterol (mmol/L)	5.35±0.18	5.48±0.10	4.2±0.11	0.5362 ^a p<0.0001 ^b
LDL-cholesterol (mmol/L)	3.40±0.17	3.72±0.09	2.73±0.10	0.975 ^a p<0.0001 ^b
HDL-cholesterol (mmol/L)				
Triglycerides (mmol/L)	2.37±0.15	1.88±0.18	0.97±0.07	p<0.0001 ^a 0.0402 ^b
Fasting glucose (mmol/L)	6.33±0.27	5.44±0.16	4.83±0.07	0.0056 ^a 0.0004 ^b
Fasting insulin (uIU/ml)	15.98±1.07	16.34±1.26	8.81±1.08	0.8281 ^a p<0.0001 ^b
HBA1c (%)	6.59±0.19	5.70±0.10	5.82±0.05	p<0.0001 ^a 0.2796 ^b
hsCRP (mg/L)	7.99±1.14	6.69±1.12	6.42±1.04	0.4205 ^a 0.8806 ^b
Medication				
Statins (%)	85	3	0	p<0.0001 ^a
Aspirin (%)	88	0	0	
Beta blockers (%)	79	0	0	

Nitrates (%)	38	0	0	
ACE-Is (%)	73	2	0	p<0.0001 ^a
Folate supplements n (%)	7 (7)	4 (4)	0	0.5395 ^b

LDL = Low density lipoprotein, HDL = High density lipoprotein, BMI = Body mass index, HbA1c = Glycated haemoglobin, hsCRP = High sensitivity C-reactive protein, ACE-Is = Angiotensin-converting-enzyme inhibitors

^a Comparison between CAD patients and Indian controls

^b Comparison between Indian and Black controls

CAD patients' characteristics according to MTHFR genotype are presented in Table 3.3. There were no significant differences observed between the wild type and variant genotypes. CAD patients harboring the variant genotypes had similar total cholesterol, LDL, HDL, BMI, etc. compared to those with the homozygous wildtype genotype.

Table 3.3 Characteristics of CAD patients according to MTHFR genotype

	MTHFR genotype		p-value
	Wild type (CC)	Variant (CT+TT)	
Diabetes n (%)	48 (61%)	15 (56%)	ns
Total cholesterol (mmol/L)	5.43±0.21	5.10±0.32	ns
LDL (mmol/L)	3.45±0.20	3.23±0.31	ns
HDL (mmol/L)	0.93±0.03	0.90±0.06	ns
Triglycerides (mmol/L)	2.45±0.18	2.13±0.26	ns
BMI (kg/m ²)	27.86±0.51	29.00±0.96	ns
Fasting glucose (mmol/L)	6.27±0.32	6.52±0.52	ns
Fasting insulin (uIU/ml)	14.99±1.18	18.87±2.37	ns
HbA1c (%)	6.45±0.22	7.00±0.38	ns
hsCRP (mg/L)	8.92±1.51	5.25±0.62	ns

4. Discussion

In the present study, we observed a significantly higher prevalence of the MTHFR 677 variant allele in CAD patients compared with Indian controls. However, no correlation between the SNP and clinical characteristics associated with CAD was found.

Similar findings were observed in a North Indian population, where the MTHFR T allele was significantly associated with CAD (Tripathi, Tewari et al. 2012). However, another study in India found a very low prevalence of the MTHFR variant allele in CAD patients (Gupta, Kotwal et al. 2012). The relationship between the MTHFR C677T SNP and CAD remains controversial, with reports in favour of and contradicting this association. Literature shows that MTHFR genotype frequencies vary amongst populations of different ethnic background (Motti, Gnasso et al. 1998; Ueland, Hustad et al. 2001; Gupta, Kotwal et al. 2012). Frequencies of the MTHFR homozygous variant genotype in control populations ranged from 1.4% in African Americans, to 5% in Dutch and Finnish, to 10% in Japanese and in Australians, to 11.5% in white populations in US (Girelli, Friso et al. 1998; Motti, Gnasso et al. 1998).

Nienaber-Rousseau et al. recently investigated the frequency of the MTHFR C677T SNP and its effect on homocysteine levels in a cohort of Black South Africans. It was observed that 15.2%, 0.8%, and 84% of the study population harboured the MTHFR 677 CT, TT, and CC genotypes respectively (Nienaber-Rousseau, Ellis et al. 2013). Subjects with the MTHFR 677 TT genotype exhibited the highest homocysteine levels while those homozygous for the wild type allele had the lowest homocysteine levels. Low prevalence of the MTHFR TT genotype among Black South Africans was speculated to confer protection to this population against risk of homocysteine associated disease. Our study supports the findings of Nienaber-Rousseau et al. with regards to the low prevalence of the MTHFR TT genotype observed amongst Black South Africans.

Nutritional status may be a reason for the differences observed in various studies, as folate deficiency may be a required condition for the detrimental consequence of the MTHFR C677T SNP on the enzyme (Farbstein and Levy 2010). Italians have among the highest ever reported frequency of the MTHFR TT genotype (considered to be a genetic marker of risk for vascular disease), but the overall prevalence of vascular complications is relatively low (Motti, Gnasso et al. 1998). This paradox could be explained by different dietary habits. Italians generally follow a Mediterranean diet, consisting of food high in folate content and therefore could counteract the effects of the MTHFR variant.

In contrast, findings from a study done on the Turkish population suggest that the MTHFR C677T SNP may be a risk for premature myocardial infarction (Gulec, Aras et al. 2001). The Turkish are

known for having low levels of plasma folate (Tokgozoglu, Alikasifoglu et al. 1999). This supports the hypothesis that the MTHFR C677T SNP increases the risk of CAD and other vascular complications in individuals with low folate levels, while in well-nourished populations it may be tolerated without detrimental consequences.

We found an association between the MTHFR 677 variant and CAD in South African Indians. The higher frequency of the MTHFR C677T SNP in SA Indians may increase their risk of CAD compared to Blacks. A larger follow up study may be required to assess the significance of this SNP.

Conflicts of interests

The authors declare that there are no conflicts of any interests.

Acknowledgements

This work was supported by the National Research Foundation and College of Health Science Scholarships.

References

- Anderson, J. L., G. J. King, et al. (1997). "A Mutation in the Methylene tetrahydrofolate Reductase Gene Is Not Associated With Increased Risk for Coronary Artery Disease or Myocardial Infarction." Journal of the American College of Cardiology **30**(5): 1206-1211.
- Andreassi, M., N. Botto, et al. (2003). "Methylene tetrahydrofolate reductase gene C677T polymorphism, homocysteine, vitamin B12, and DNA damage in coronary artery disease." Human Genetics **112**(2): 171-177.
- Clarke, R. (1998). "Lowering blood homocysteine with folic acid based supplements: meta-analysis of randomised trials. Homocysteine Lowering Trialists' Collaboration." BMJ **316**(0959-8138 (Print)): 894-898.
- Clarke, R., D. A. Bennett, et al. (2012). "Homocysteine and Coronary Heart Disease: Meta-analysis of MTHFR Case-Control Studies, Avoiding Publication Bias." PLoS Med **9**(2): e1001177.
- Coffey, M., G. K. Crowder, et al. (2003). "Reducing Coronary Artery Disease by Decreasing Homocysteine Levels." Critical Care Nurse **23**(1): 25-30.
- Doshi, S. N., I. F. W. McDowell, et al. (2002). "Folic Acid Improves Endothelial Function in Coronary Artery Disease via Mechanisms Largely Independent of Homocysteine Lowering." Circulation **105**(1): 22-26.
- Farbstein, D. and A. P. Levy (2010). "The Genetics of Vascular Complications in Diabetes Mellitus." Cardiology Clinics **28**(3): 477-496.
- Friso, S., D. Girelli, et al. (2002). "A1298C methylene tetrahydrofolate reductase mutation and coronary artery disease: relationships with C677T polymorphism and homocysteine/folate metabolism." Clinical and Experimental Medicine **2**(1): 7-12.
- Girelli, D., S. Friso, et al. (1998). "Methylene tetrahydrofolate Reductase C677T Mutation, Plasma Homocysteine, and Folate in Subjects From Northern Italy With or Without Angiographically Documented Severe Coronary Atherosclerotic Disease: Evidence for an Important Genetic-Environmental Interaction." Blood **91**: 4158-4163.
- Girija, G. (1997). Cardiovascular Disease Prevention - American Heart Association Heart and Stroke Statistical Update. **26**: 78-83.
- Gudnason, V., D. Stansbie, et al. (1998). "C677T (thermolabile alanine/valine) polymorphism in methylene tetrahydrofolate reductase (MTHFR): its frequency and impact on plasma homocysteine concentration in different European populations." Atherosclerosis **136**(2): 347-354.
- Gulec, S., O. Aras, et al. (2001). "Methylene tetrahydrofolate Reductase Gene Polymorphism and Risk of Premature Myocardial Infarction." Clinical Cardiology **24**: 281-284.

- Gupta, S. K., J. Kotwal, et al. (2012). "Role of homocysteine & MTHFR C677T gene polymorphism as risk factors for coronary artery disease in young Indians." Indian Journal of Medical Research **135**(4): 506-512.
- Hughes, K., K. C. Lun, et al. (1990). "Cardiovascular diseases in Chinese, Malays, and Indians in Singapore. I. Differences in mortality." Journal of Epidemiology and Community Health **44**(1): 24-28.
- Kluijtmans, L. A. J. and A. S. Whitehead (2001). "Methylenetetrahydrofolate reductase genotypes and predisposition to atherothrombotic disease." European Heart Journal **22**: 294-299.
- Lal, S. (2004). Genetic epidemiology of coronary artery disease. Master of Science, National University of Singapore.
- Mehlig, K., K. Leander, et al. (2013). "The association between plasma homocysteine and coronary heart disease is modified by the MTHFR 677C>T polymorphism." Heart.
- Motti, C., A. Gnasso, et al. (1998). "Common mutation in methylenetetrahydrofolate reductase. Correlation with homocysteine and other risk factors for vascular disease." Atherosclerosis **139**: 377-383.
- Muskiet, F. A. J. (2005). "The importance of (early) folate status to primary and secondary coronary artery disease prevention." Reproductive Toxicology **20**(3): 403-410.
- Nienaber-Rousseau, C., S. M. Ellis, et al. (2013). "Gene–environment and gene–gene interactions of specific MTHFR, MTR and CBS gene variants in relation to homocysteine in black South Africans." Gene **530**(1): 113-118.
- Norman R BD, S. M., Pieterse D, Groenwald P. (2006). Revised Burden of Disease Estimates for the comparative risk factor assessment, South Africa 2000. Cape Town: Medical Research Council 2006.
- Rajadurai, J., J. Arokiasamy, et al. (1992). "Coronary artery disease in Asians." Australian and New Zealand Journal of Medicine **22**(4): 345-348.
- Rajeshwari, R., T. A. Nicklas, et al. (2005). "Cardiovascular diseases—a major health risk in Asian Indians." Nutrition research (New York, N.Y.) **25**(6): 515-533.
- Ranjith, N., R. J. Pegoraro, et al. (2005). "Demographic data and outcome of acute coronary syndrome in the South African Asian Indian population." Cardiovascular Journal of South Africa **16**(1): 48-54.
- Rissam, H. S., S. Banzal, et al. Profile of acute myocardial infarction in Indians in Kingdom of Saudi Arabia (unpublished data).
- Rissam, H. S., S. Kishore, et al. (2001). "Coronary Artery Disease in Young Indians - The Missing Link." Journal, Indian Academy of Clinical Medicine **2**(3).
- Sambrook, J. and D. W. Russell (2001). Rapid isolation of mammalian DNA in Molecular Cloning: A laboratory manual. New York, COLD SPRING HARBOR LABORATORY PRESS.

- Sastry, B. K. S., J. Golla, et al. (2011). "Genetic interactions between MTHFR (C677T), methionine synthase (A2756G, C2758G) variants with vitamin B12 and folic acid determine susceptibility to premature coronary artery disease in Indian population." Journal of Cardiovascular Disease Research **2**(3): 156-163.
- Seedat, Y. K. (2005). "Review: Diabetes mellitus in South African Indians." The British Journal of Diabetes & Vascular Disease **5**(5): 249-251.
- Sharma M, G. M. (2005). "Premature coronary artery disease in Indians and its associated risk factors." Vascular Health and Risk Management **1**(3): 217-225.
- Stanger, O., H.-J. Semmelrock, et al. (2002). "Effects of Folate Treatment and Homocysteine Lowering on Resistance Vessel Reactivity in Atherosclerotic Subjects." Journal of Pharmacology and Experimental Therapeutics **303**(1): 158-162.
- Sun, J., Y. Xu, et al. (2005). "Methylenetetrahydrofolate reductase polymorphism associated with susceptibility to coronary heart disease in Chinese type 2 diabetic patients." Molecular and Cellular Endocrinology **229**(1-2): 95-101.
- Tokgozoglu, S. L., M. Alikasifoglu, et al. (1999). "Methylenetetrahydrofolate reductase genotype and the risk and extent of coronary artery disease in a population with low plasma folate." Heart **81**: 518-522.
- Tripathi, R., S. Tewari, et al. (2012). "Biomolecular Marker Screener for Coronary Artery Disease in Indian perspective." Der Pharmacia Lettre **4**(1): 61-66.
- Ueland, P. M., S. Hustad, et al. (2001). "Biological and clinical implications of the MTHFR C677T polymorphism." Trends in Pharmacological Sciences **22**(4): 194-201.
- Vasisht, S., R. Gulati, et al. (2002). "Polymorphism (C677T) in the 5,10-Methylenetetrahydrofolate reductase (MTHFR) Gene: A Preliminary Study in North Indian Men." Indian Journal of Clinical Biochemistry **17**(1): 99-107.
- Wainwright, J. (1966). "Cardiovascular disease in the Asiatic (Indian) population of Durban." South African medical journal **43**(6): 136-138.

CHAPTER 4

Sirtuin 1 rs1467568 and rs7895833 in South African Indians with Early-onset Coronary Artery Disease

Prithiksha Ramkaran, Alisa Phulukdaree, Sajidah Khan, Devapregasan Moodley, Anil A. Chaturgoon

Discipline of Medical Biochemistry and Chemical Pathology, Howard College Campus, University of KwaZulu-Natal, Durban, South Africa

Prithiksha Ramkaran, BMedSc Hons

Devapregasan Moodley, PhD

Anil A. Chaturgoon, PhD

Department of Physiology, School of Medicine, Faculty of Health Sciences, Prinshof Campus, University of Pretoria, South Africa

Alisa Phulukdaree, PhD

Department of Cardiology, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa

Sajidah Khan, MB ChB, FCP (SA), PhD

Corresponding author:

Professor Anil A. Chaturgoon

School of Laboratory Medicine and Medical Sciences

Discipline of Medical Biochemistry and Chemical Pathology

George Campbell Building – South Entrance, 3rd Floor

Kind George V Avenue

Howard College Campus

University of KwaZulu-Natal

Durban, South Africa

E-mail: chatur@ukzn.ac.za,

Tel: +2731 260 4404

CardioVascular Journal of Africa (2015) (*In press*)

Manuscript reference number: CVJSA-D-15-00055

Abstract

Background: Sirtuin 1 (SIRT1), a class III histone deacetylase, has been identified as a candidate molecule affecting the epigenetic mechanisms of cardiovascular disease (CVD). Previous studies have shown that some SIRT 1 single nucleotide polymorphisms (SNPs) are associated with body mass index, diabetes, blood pressure, cholesterol metabolism, and coronary artery calcification. We investigated two A>G SIRT1 SNPs, rs1467568 and rs7895833, in young South African (SA) Indians with coronary artery disease (CAD) and compared them to Indian and Black controls.

Methods: For rs1467568, a total of 287 subjects were recruited into this study (104 CAD patients, 99 age-, sex-, and race-matched controls, and 84 age- and sex-matched Black controls). For rs7895833, a total of 281 subjects were recruited into this study (100 CAD patients, 99 age-, sex-, and race-matched controls, and 82 age- and sex-matched Black controls). All patients were male, of Indian ethnicity, confirmed on angiography, mean age 37.5; range 24-45 years. All subjects were genotyped using TaqMan SNP Genotyping Assays.

Results: The variant allele for both SNPs was found at a higher frequency in the total Indian group compared to the total Black population (rs1467568: 41% vs. 18.5% respectively, $p < 0.0001$, OR=3.190 95% CI 2.058 – 4.0943 and rs7895833: 41% vs. 22% respectively, $p < 0.0001$, OR=2.466 95% CI 1.620 – 3.755). Indian controls presented with a higher frequency for both SNPs compared to Black controls (rs1467568: 40% vs. 18.5% respectively, $p < 0.0001$, OR=2.996 95% CI 1.850 – 4.853 and rs7895833: 41% vs. 22% respectively, $p < 0.0001$, OR=2.513 95% CI 1.578 – 4.004). No difference was seen in the distribution of both SNPs between CAD patients and either control group. We did not observe any association between the SNPs and clinical parameters in CAD patients and controls.

Conclusion: Both SNP variant alleles occur more frequently in SA Indians than in SA Blacks. A larger study group and further analysis is required to assess whether these SIRT1 SNPs may serve as risk factors that contribute to Indians developing early onset CAD.

Keywords:

Sirtuin 1, rs1467568, rs7895833, single-nucleotide polymorphism, premature coronary artery disease, South African Indians

Introduction

Sirtuins are a class of NAD⁺-dependent proteins involved in a wide range of biological processes such as aging, transcription, apoptosis, and inflammation.¹ Sirtuin 1 (SIRT1) is located in the nucleus and the cytoplasm, and plays an important role in epigenetic regulation by deacetylating a range of transcription factors to control downstream gene expression.² The targets of SIRT1 include Forkhead box O (FOXO)1, FOXO3, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), tumour suppressor p53, nuclear factor-kappa B (NF- κ B), Notch, hypoxia-inducible factor (HIF)1 α , liver X receptor (LXR), farnesoid X receptor (FXR), and sterol regulatory element-binding protein (SREBP)1c.³

Recent studies have demonstrated a protective role of SIRT1 in atherosclerosis, the underlying process of coronary artery disease (CAD).⁴ SIRT1 performs an anti-inflammatory function by downregulating the expression of several pro-inflammatory cytokines by interfering with the NF- κ B signalling pathway. By deacetylating NF- κ B, SIRT1 suppresses the expression of lectin-like oxidised low-density lipoprotein receptor-1 (Lox-1), a scavenger receptor for oxidised low-density lipoproteins (oxLDL), therefore preventing foam cell formation.⁴ SIRT1 controls the activity of LXR, and important regulator of lipid homeostasis and inflammation.⁴ Activation of LXR results in expression of ATP-binding cassette (ABC) transporter ABCA1 that regulates the removal of cholesterol into high density lipoproteins (HDL), a process known as reverse cholesterol transport (RCT). Dysfunctional RCT could lead to accumulation of cholesterol, thus stimulating foam cell production and the progression of atherosclerosis.^{4, 5} Given the important role of SIRT1 in cardiovascular disease, research on genetic variation in the SIRT1 gene has become of interest.

Genetic variations such as single nucleotide polymorphisms (SNPs) in the SIRT1 gene has been associated with inflammation, body mass index, type 2 diabetes, blood pressure, and dyslipidemia, all of which are well-established risk factors for CAD.^{2, 6-9} Coronary artery disease remains a leading cause of mortality worldwide, with an unusually high prevalence of early-onset disease among the Indian population. South African (SA) Indians have a much higher prevalence of CAD compared to SA Blacks.¹⁰ There are currently no studies on SIRT1 SNPs in SA Indians with CAD. We therefore investigated the SIRT1 A>G SNPs, rs1467568 and rs7895833, in young SA Indians with CAD and compared them to Indian and Black controls.

Materials and Methods

Patient recruitment

SIRT1 rs1467568

A total of 287 subjects were recruited into this study (104 CAD patients, 99 age-, sex-, and race-matched controls, and 84 age- and sex-matched Black controls) following institutional ethical approval (BE067/14).

SIRT1 rs7895833

A total of 281 subjects were recruited into this study (100 CAD patients, 99 age-, sex-, and race-matched controls, and 82 age- and sex-matched Black controls) following institutional ethical approval (BE067/14).

The inclusion criteria for CAD patients were: Indian ancestry and unrelated, adult males aged <45 years, and stable CAD confirmed on angiography. The exclusion criteria for controls included an acute coronary syndrome/revascularisation procedure in the preceding 3 months, chronic renal or liver disease, malignancy and known inflammatory or infectious disease.

Clinical markers

Blood samples were obtained following an overnight fast. A full pathology report of clinical markers was assessed by routine laboratory testing at Global Clinical and Viral Laboratory (Durban, South Africa), a South African National Accreditation System (SANAS) certified laboratory. The following parameters were tested: Haematology (Roche Sysmex 1800XT), Chemistry (Beckman Coulter DXC600), Endocrinology and hsCRP (Siemens Centaur XP), Serology (BD Biosciences FACS Calibur), as per international standards to obtain levels of total cholesterol, HDL, LDL, triglycerides, fasting glucose, 2-h glucose, fasting insulin, glycosylated haemoglobin, sodium, potassium, bicarbonate, chloride, urea, creatinine, glomerular filtration rate, CD4 count, CD8 count, CD45 count and CD3 count. The physical measurements of weight, height, abdominal circumference, waist circumference and patient history were conducted by the cardiologist (Dr. S. Khan).

DNA Extraction

Genomic DNA was extracted from the whole blood sample of each patient and control, according to the method described by Sambrook et al.¹¹ Cells were transferred to 600 µl lysis buffer (0.5 % sodium dodecyl sulphate (SDS), 150 mM NaCl, 10 mM ethylenediaminetetra-acetic acid (EDTA), 10 mM Tris-HCl (pH 8.0)). To this, RNase A (100 µg/ml; DNase-free) was added to the solution and incubated (37 °C, 1 h). Proteinase K (200 µg/ml) was then added and incubated (3 h, 50 °C). Protein contaminants were then precipitated by adding 5 mM 0.1 % potassium acetate before centrifugation (5,000×g; 15 min). Supernatants containing genomic DNA were transferred to fresh tubes and extracted with 100 % isopropanol on ice, and thereafter washed with 70 % ethanol. DNA samples were dissolved in 10 mM Tris and 0.1 mM EDTA (pH 7.4, 4 °C). DNA concentration was determined using the Nanodrop2000 spectrophotometer, and all samples were standardised to a concentration of 10 ng/µl.

Genotyping

Following the manufacturer's protocol, TaqMan[®] SNP Predesigned Genotyping Assay (Life Technologies, Cat #4351379) was used to genotype all subjects for both SNPs.

The TaqMan Predesigned Genotyping Assay contains two primers for amplifying the sequence of interest and two TaqMan[®] minor-groove binding (MGB) probes for detecting alleles. The presence of two probe pairs in each reaction allows genotyping of the two possible variant alleles at the SNP site in a DNA target sequence. The genotyping assay determines the presence or absence of a SNP based on the change in fluorescence of the dyes associated with the probes. The TaqMan[®] MGB Probes consist of target-specific oligonucleotides with a reporter dye at the 5' end of each probe: One VIC[®]-labelled probe to detect Allele 1 sequence (A-allele in the case of rs1467568 and rs7895833) and one FAM[™] labelled probe to detect Allele 2 sequence (G-allele in the case of rs1467568 and rs7895833). A fluorescence signal for both dyes indicates heterozygous for allele 1-allele 2 (AG).

A final reaction mixture consisted of 40× TaqMan[®] Predesigned Genotyping Assay, 2X TaqMan[®] Genotyping Master Mix, nuclease-free water, and a 10ng genomic DNA template. The experiment was done using the Applied Biosystems[®] ViiA[™] 7 Real-Time PCR System.

Statistical analysis

The Hardy–Weinberg equilibrium was used to test for deviation of allele/genotype frequency. All other statistical analysis was performed with Graphpad prism software (version 5.0). Allele and genotype frequencies were calculated using the Fisher's exact and Chi square tests, respectively. The comparison of biochemical measures between the wildtype and variant genotypes were done by a nonparametric t test. Results are expressed as mean \pm standard error. A p value less than 0.05 was considered statistically significant.

Results

SIRT1 rs1467568

The genotype distribution complied with the Hardy-Weinberg equilibrium in CAD patients and Indian controls (Chi squared $p=0.233$ and $p=0.941$, respectively), but not in the Black control group (Chi squared $p<0.05$).

No significant difference was observed in the distribution of the SIRT1 rs1467568 alleles between CAD patients and Indian controls (41% vs. 40% respectively, $p=0.9196$, OR = 1.040 95% CI 0.6998-1.545). Indian controls presented with a higher frequency of the variant allele compared to the Black controls (40% vs. 18.5% respectively, $p<0.0001$, OR=2.996 95% CI 1.850 – 4.853). The variant allele was found at a higher frequency in the total Indian group compared to the total Black population (41% vs. 18.5% respectively, $p<0.0001$, OR=3.057 95% CI 1.974 – 4.733) (Table 4.1).

SIRT1 rs7895833

The genotype distribution complied with the Hardy-Weinberg equilibrium in CAD patients, Indian controls, and Black controls (Chi squared $p=0.970$ and $p=1.000$, and $p=0.164$ respectively).

No significant difference was observed in the distribution of the SIRT1 rs7895833 alleles between CAD patients and Indian controls (40.5% vs. 41% respectively, $p=0.9188$, OR=0.9629 95% CI 0.6457-1.436). Indian controls presented with a higher frequency of the variant allele compared to the Black controls (41% vs. 22% respectively, $p<0.0001$, OR=2.513 95% CI 1.578 – 4.004). The variant

allele was found at a higher frequency in the total Indian group compared to the total Black population (41% vs. 22% respectively, $p < 0.0001$, $OR = 2.466$ 95% CI 1.620 – 3.755) (Table 4.1).

Table 4.1 SIRT1 rs1467568 and rs7895833 genotype and allele frequencies in CAD patients and controls

SIRT1 rs1467568	CAD Patients n=104	Indian Controls n=99	Total Indians n=203	SA	Black Controls n=84
Genotypes					
AA	40 (38.46%)	36 (36.36%)	76 (37%)		62 (73.81%)
AG	42 (40.38%)	46 (46.46%)	88 (43%)		13 (15.48%)
GG	22 (21.15%)	17 (17.17%)	39 (19%)		9 (10.71%)
Alleles					
A	122 (59%)	118 (60%)	240 (59%)		137 (81.5%)
G	86 (41%)	80 (40%)	166 (41%)		31 (18.5)
SIRT1 rs7895833	CAD Patients n=100	Indian Controls n=99	Total Indians n=199	SA	Black Controls n=82
Genotypes					
AA	36 (36%)	34 (34.34%)	70 (35%)		47 (57.32%)
AG	47 (47%)	48 (48.48%)	95 (48%)		34 (41.46%)
GG	17 (17%)	17 (17.17%)	34 (17%)		1 (1.22%)
Alleles					
A	119 (59.5%)	116 (59%)	235 (59%)		128 (78%)
G	81 (40.5%)	82 (41%)	163 (41%)		36 (22%)

Characteristics of study participants were reported previously.^[12] As expected, CAD patients presented with more conventional risk factors (higher body mass index [BMI], total and LDL cholesterol, triglycerides, and a higher prevalence of type 2 diabetes mellitus) than the control groups. No association between the SIRT1 SNPs and biochemical measures were found in CAD patients (Table 4.2), Indian controls (Table 4.3) and Black controls (Table 4.4).

Table 4.2 Characteristics of CAD patients according to the SIRT1 rs1467568 and SIRT1 rs7895833 genotypes

	SIRT1 rs1467568 genotype		p-value	SIRT1 rs7895833 genotype		p-value
	Wild type (AA)	Variant (AG+GG)		Wild type (AA)	Variant (AG+GG)	
BMI (kg/m ²)	27.52±0.81	28.57±0.55	ns	28.02±0.80	28.33±0.59	ns
Total cholesterol (mmol/L)	5.73±0.32	5.17±0.20	ns	5.32±0.24	5.46±0.25	ns
LDL (mmol/L)	3.70±0.29	3.27±0.21	ns	3.41±0.23	3.47±0.24	ns
HDL (mmol/L)	0.98±0.04	0.89±0.03	ns	0.91±0.04	0.93±0.04	ns
Triglycerides (mmol/L)	2.41±0.28	2.37±0.18	ns	2.34±0.24	2.38±0.20	ns
Fasting glucose (mmol/L)	6.48±0.50	6.27±0.33	ns	6.18±0.47	6.32±0.34	ns
Fasting insulin (uIU/ml)	16.97±2.21	15.54±1.12	ns	14.17±1.19	16.95±1.61	ns
HBA1c (%)	6.63±0.33	6.61±0.24	ns	6.57±0.34	6.60±0.24	ns
hsCRP (mg/L)	9.83±2.58	6.97±0.98	ns	8.93±2.42	7.78±1.31	ns
IL-6 (pg/mL)	2.80±0.90	2.45±0.59	ns	2.41±0.80	2.73±0.68	ns

Table 4.3 Characteristics of Indian controls according to the SIRT1 rs1467568 and SIRT1 rs7895833 genotype

	SIRT1 rs1467568 genotype		p-value	SIRT1 rs7895833 genotype		p-value
	Wild type (AA)	Variant (AG+GG)		Wild type (AA)	Variant (AG+GG)	
BMI (kg/m ²)	25.88±0.93	26.65±0.69	ns	25.14±1.01	26.88±0.66	ns
Total cholesterol (mmol/L)	5.32±0.16	5.54±0.13	ns	5.56±0.19	5.41±0.12	ns
LDL (mmol/L)	3.47±0.13	3.86±0.12	ns	3.88±0.17	3.63±0.11	ns
HDL (mmol/L)	1.04± 0.07	0.91±0.03	ns	0.97±0.07	0.95±0.03	ns
Triglycerides (mmol/L)	1.79±0.22	1.92±0.27	ns	1.63±0.17	2.00±0.27	ns
Fasting glucose (mmol/L)	5.59±0.34	5.38±0.16	ns	5.27±0.20	5.56±0.22	ns
Fasting insulin (uIU/ml)	15.91±1.96	16.72±1.66	ns	13.36±1.46	18.03±1.75	ns
HBA1c (%)	5.78±0.21	5.65±0.11	ns	5.85±0.16	5.63±0.13	ns
hsCRP (mg/L)	4.58±0.60	7.95±1.71	ns	6.52±1.41	6.87±1.57	ns
IL-6 (pg/mL)	2.16±0.79	2.86±0.63	ns	2.83±0.87	2.48±0.60	ns

Table 4.4 Characteristics of Black controls according to the SIRT1 rs1467568 and SIRT1 rs7895833 genotype

	SIRT1 rs1467568 genotype		p-value	SIRT1 rs7895833 genotype		p-value
	Wild type (AA)	Variant (AG+GG)		Wild type (AA)	Variant (AG+GG)	
BMI (kg/m ²)	25.53±0.54	27.13±1.20	ns	25.58±0.63	26.57±0.87	ns
Total cholesterol (mmol/L)	4.12±0.12	4.47±0.23	ns	4.30±0.13	4.14±0.18	ns
LDL (mmol/L)	2.62±0.10	3.02±0.22	ns	2.78±0.12	2.71±0.16	ns
HDL (mmol/L)	1.05± 0.045	1.03±0.088	ns	1.08±0.05	0.99±0.06	ns
Triglycerides (mmol/L)	0.99±0.072	0.93±0.15	ns	0.98±0.08	0.96±0.11	ns
Fasting glucose (mmol/L)	4.80± 0.084	4.90±0.11	ns	4.87±0.11	4.77±0.08	ns
Fasting insulin (uIU/ml)	7.69±0.67	12.11±3.74	ns	9.21±1.73	8.59±1.10	ns
HBA1c (%)	5.83±0.062	5.79±0.082	ns	5.87±0.07	5.76±0.077	ns
hsCRP (mg/L)	6.64±1.86	5.82±1.23	ns	7.81±2.42	4.83±0.84	ns

BMI = body mass index, LDL = Low density lipoprotein, HDL = High density lipoprotein, HBA1c = Glycated haemoglobin, hsCRP = High sensitivity C-reactive protein, IL-6 = Interleukin-6, ns = non-significant

Discussion

Indian populations throughout the world show early-onset CAD, one to two decades earlier than other ethnic groups.¹³ South African Indians have the highest mortality rates due to CAD, while Black South Africans have a very low prevalence of the disease.¹⁰

Increasing evidence has shown that SIRT1 is involved in CAD by regulating a number of key metabolic and physiological processes. Sirtuin 1 serves as an anti-atherosclerotic factor by mediating endothelial nitric oxide synthase (eNOS) and improving endothelial dysfunction, regulating inflammation, reversing cholesterol transport and reducing risk of CAD.¹⁴

Several SNPs have been identified in SIRT1, a candidate molecule involved in the epigenetic regulation of CAD. To date, there are only a few human genetic association studies regarding SIRT1 SNPs and CAD. The present study was the first investigation of SIRT1 rs1467568 and rs7895833 in SA Indian CAD patients. We observed that the variant alleles of both SIRT1 SNPs occurred more frequently in SA Indians compared to SA Blacks. We did not observe any difference in allele frequencies between CAD patients and control groups.

Previous studies showed that some of the SIRT1 SNPs are associated with BMI and obesity, glucose tolerance and diabetes, blood pressure, cholesterol metabolism and coronary artery calcification, all of which contribute to CAD phenotype.¹⁵⁻¹⁹ We examined the possible association between rs1467568 and rs7895833 in SIRT1 and BMI, total cholesterol, LDL, HDL, triglycerides, fasting glucose, fasting insulin, HbA1c, hsCRP, or IL-6 in CAD patients and control groups, but did not observe any association.

The Rotterdam study investigated SIRT1 variation (assessed by three tagging SIRT1 SNPs: rs7895833, rs1467568, and rs497849) in relation to BMI and risk of obesity in 4573 participants, including 413 individuals with prevalent and 378 with incident type 2 diabetes mellitus (T2DM).²⁰ In subjects with prevalent T2DM, homozygous carriers the SIRT1 haplotype 1 had 1.9 times (95% CI 1.1-3.2) increased risk of CVD mortality compared to non-carriers. An intended replication study (Erasmus Rucphen Family study) was carried out involving 2347 participants. Both studies observed that the minor alleles of rs7895833 (G allele) and rs1467568 (A allele) were associated with lower BMI and a 13-18% decreased risk of obesity in two independent Dutch populations.¹⁷ In another study, the A allele of rs7895833 was associated with increased risk of obesity and hypertension in Japanese men.¹⁵

Recent studies investigated the association between SIRT1 SNPs (rs7895833, rs7069102, rs144124002 and rs2273773) and CAD in a Turkish population. While rs7069102, rs2273773 and rs144124002 were significantly associated with increased risk for CAD, they found no association between rs7895833 and CAD.^{21,22}

Shimoyama et al. reported that SIRT1 rs7069102 and rs2273773 are associated with abnormal cholesterol metabolism and coronary artery calcification, respectively, in Japanese haemodialysis (HD) patients. The study also found that the A allele frequency of SIRT1 rs7895833 and G allele frequency of rs7069102 were significantly lower in HD patients compared to controls, suggesting an impact on survival.²³

The allele frequency of rs7895833 and rs1467568 show ethnic variation, and is a possible reason for differing disease patterns among populations. The frequency of the rs7895833 A allele was relatively low (0.29) in Japanese compared to Dutch, Turkish and Caucasians who had similar allele frequencies (0.80, 0.85, and 0.80 respectively).^{15, 17, 24} The A allele of rs1467568 (reported as the protective allele) showed marked difference in frequency between the European (0.25) and Japanese (0.84).²⁴

Conclusion

Both SNP variant alleles occur more frequently in SA Indians than in SA Blacks, but no difference was found between CAD patients and controls. The present study is limited by sample size and a larger study may be required to fully assess the functional significance of these polymorphisms.

Acknowledgments

P. Ramkaran thanks the National Research Foundation (NRF) for a scholarship and UKZN (College of Health Sciences) for funding this study.

Author Disclosure Statement

No competing financial interests exist.

References:

1. Preyat, N. and O. Leo, *Sirtuin deacylases: a molecular link between metabolism and immunity*. Journal of Leukocyte Biology, 2013. **93**(5): p. 669-680.
2. Cui, Y., et al., *Genetic analysis of the SIRT1 gene promoter in myocardial infarction*. Biochemical and Biophysical Research Communications, 2012. **426**(2): p. 232-236.
3. Morris, B.J., *Seven sirtuins for seven deadly diseases of aging*. Free Radical Biology and Medicine, 2013. **56**(0): p. 133-171.
4. Stein, S. and C.M. Matter, *Protective roles of SIRT1 in atherosclerosis*. Cell Cycle, 2011. **10**(4): p. 640-647.
5. Li, X., et al., *SIRT1 Deacetylates and Positively Regulates the Nuclear Receptor LXR*. Molecular Cell, 2007. **28**(1): p. 91-106.
6. Finkel, T., C.-X. Deng, and R. Mostoslavsky, *Recent progress in the biology and physiology of sirtuins*. Nature, 2009. **460**(7255): p. 587-591.
7. Haigis, M.C. and D.A. Sinclair, *Mammalian Sirtuins: Biological Insights and Disease Relevance*. Annual Review of Pathology: Mechanisms of Disease, 2010. **5**(1): p. 253-295.
8. Horio, Y., et al., *Cellular and molecular effects of sirtuins in health and disease*. Clinical Science, 2011. **121**(5): p. 191-203.
9. Houtkooper, R.H., E. Pirinen, and J. Auwerx, *Sirtuins as regulators of metabolism and healthspan*. Nat Rev Mol Cell Biol, 2012. **13**(4): p. 225-238.
10. Norman R BD, S.M., Pieterse D, Groenwald P., *Revised Burden of Disease Estimates for the comparative risk factor assessment, South Africa 2000*. Cape Town: Medical Research Council 2006., 2006.
11. Sambrook, J. and D.W. Russell, *Rapid isolation of mammalian DNA in Molecular Cloning: A laboratory manual*. 3 ed2001, New York: COLD SPRING HARBOR LABORATORY PRESS.
12. Phulukdaree, A., et al., *GST polymorphisms and early-onset coronary artery disease in young South African Indians*. SAMJ: South African Medical Journal, 2012. **102**(7): p. 627-630.
13. Sharma M, G.M., *Premature coronary artery disease in Indians and its associated risk factors*. Vascular Health and Risk Management, 2005. **1**(3): p. 217-225.

14. Ma, L. and Y. Li, *SIRT1: Role in cardiovascular biology*. Clinica Chimica Acta, 2015. **440**(0): p. 8-15.
15. Shimoyama, Y., et al., *Sirtuin 1 gene polymorphisms are associated with body fat and blood pressure in Japanese*. Translational Research, 2011. **157**(6): p. 339-347.
16. Peeters, A., et al., *Association of SIRT1 gene variation with visceral obesity*. Human Genetics, 2008. **124**(4): p. 431-436.
17. Zillikens, M.C., et al., *SIRT1 Genetic Variation Is Related to BMI and Risk of Obesity*. Diabetes, 2009. **58**(12): p. 2828-2834.
18. Botden, I.P.G., et al., *Variants in the SIRT1 Gene May Affect Diabetes Risk in Interaction With Prenatal Exposure to Famine*. Diabetes Care, 2012. **35**(2): p. 424-426.
19. Shimoyama, Y., et al., *SIRTUIN 1 gene polymorphisms are associated with cholesterol metabolism and coronary artery calcification in Japanese hemodialysis patients*. Journal of Renal nutrition, 2012. **22**(1): p. 114-119.
20. Zillikens, M.C., et al., *SIRT1 genetic variation and mortality in type 2 diabetes: interaction with smoking and dietary niacin*. Free Radical Biology and Medicine, 2009. **46**(6): p. 836-841.
21. İzmirli, M., et al., *The effect of the SIRT1 2827 A>G polymorphism, resveratrol, exercise, age and occupation in Turkish population with cardiovascular disease*. The Anatolian Journal of Cardiology, 2014. **14**.
22. Kilic, U., et al., *SIRT1 Gene Polymorphisms Affect the Protein Expression in Cardiovascular Diseases*. PLoS ONE, 2014. **9**(2): p. e90428.
23. Shimoyama, Y., et al., *SIRTUIN 1 Gene Polymorphisms are Associated With Cholesterol Metabolism and Coronary Artery Calcification in Japanese Hemodialysis Patients*. Journal of Renal nutrition, 2012. **22**(1): p. 114 - 119.
24. Maeda, S., et al., *Association between single nucleotide polymorphisms within genes encoding sirtuin families and diabetic nephropathy in Japanese subjects with type 2 diabetes*. Clinical and Experimental Nephrology, 2011. **15**(3): p. 381-390.

CHAPTER 5

MicroRNA-499 Single Nucleotide Polymorphism in South African Indians with early-onset Coronary Artery Disease

P Ramkaran,¹ BMedSc Hons; A Phulukdaree,² PhD; S Khan,³ MBChB, FCP(SA), PhD; D Moodley,¹ PhD; A A Chuturgoon,¹ PhD

¹Discipline of Medical Biochemistry and Chemical Pathology, School of Laboratory Medicine and Medical Sciences, Howard College Campus, University of KwaZulu-Natal, Durban, South Africa

²Department of Physiology, School of Medicine, Faculty of Health Sciences, Prinshof Campus, University of Pretoria, South Africa

³Department of Cardiology, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa

P Ramkaran: prithiksharamkaran@gmail.com

A Phulukdaree: Alisa.Phulukdaree@up.ac.za

S Khan: SajidahKha@ialch.co.za

D Moodley: Devan_Moodley@hms.harvard.edu

Correspondence to:

Professor Anil A. Chuturgoon

School of Laboratory Medicine and Medical Sciences

Discipline of Medical Biochemistry and Chemical Pathology

George Campbell Building – South Entrance, 3rd Floor

King George V Avenue

Howard College Campus

University of KwaZulu-Natal

Durban

South Africa

Tel: +27 31 260 4404

E-mail address: CHUTUR@ukzn.ac.za

Manuscript submitted to *South African Heart Journal* (2015)

ABSTRACT

Background: MicroRNA (miR)-499 is an inflammatory-associated miR implicated in chronic diseases such as coronary artery disease (CAD) which affects South Africans of Indian ethnicity.

Objectives: To report the frequency of the miR-499 A>G single nucleotide polymorphism (SNP) (rs3746444) in young South African (SA) Indians and Blacks, and investigate its association with CAD.

Methods: A total of 289 subjects were recruited into this case-control study which included 105 CAD patients (diagnosed on angiography, mean age 37.5; range 24-45 years), 100 age-, sex-, and race-matched controls, and 84 age- and sex-matched Black controls. Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP) was used to genotype all subjects. Interleukin (IL)-6 was measured by ELISA, and all other biochemical markers were provided by pathology reports.

Results: The miR-499 variant allele was found at a higher frequency in the total Indian group (34%) compared to the total Black population (22%) ($p=0.0070$, OR=1.796 95% CI 1.182 – 2.730). Indian cases presented with higher frequency of the variant allele compared to Indian controls (38% vs. 29%, $p = 0.059$, respectively). Higher levels of hsCRP and IL-6 were observed in CAD patients with the variant genotypes compared to those with the wild type genotypes (8.92 ± 1.91 vs. 6.73 ± 0.87 mg/L; $p=0.299$, 3.02 ± 0.77 vs. 2.18 ± 0.57 pg/mL; $p=0.381$ respectively).

Conclusion: The higher frequency of rs3746444 variant in SA Indians may be a contributing factor to the higher risk profile for the development of premature CAD in Indians. The A-to-G change in miR-499 seed sequence may interfere with its binding to and interaction with targets, thereby regulating inflammation in CAD patients.

Key words: microRNA-499, Single nucleotide polymorphism, rs3746444, early-onset coronary artery disease, South African Indians

INTRODUCTION

Coronary artery disease (CAD) is a multifactorial chronic inflammatory disease depending on the complex interaction amongst environmental and lifestyle associated risk factors, and several predisposing genes.^[1] On a genetic level, single nucleotide polymorphisms (SNPs) in key genes may play a role in an individual's susceptibility to the manifestation of the disease. The role of SNPs in protein coding genes has been well investigated, but remains poorly understood in microRNAs (miRs).

MicroRNAs are a class of small noncoding RNA molecules that negatively regulate gene expression of target genes by inhibiting messenger RNA (mRNA) translation or promoting mRNA degradation.^[2, 3] A single miR is able to regulate multiple target genes whereas one target gene may be regulated by various miRs.^[4] MicroRNAs regulate most biological processes; therefore aberrant miR expression is likely to be implicated in several pathological conditions.^[5, 6] Genetic variation such as SNPs in miRs may affect their processing, expression and interaction with target genes.^[4] Altered miR expression has been well investigated in various diseases^[7, 8] but miRs in cardiovascular disease is still an emerging field of research with very few studies focusing on coronary artery disease.^[9]

We recently showed that a common SNP in miR-146a (rs2910164) influences the expression of mature miR-146a in CAD patients. MicroRNA-146a primarily targets interleukin-1 receptor-associated kinase 1 (IRAK-1) and tumour necrosis factor receptor associated factor 6 (TRAF-6), which results in inhibition of nuclear factor kappa-B (NF- κ B) via the toll-like receptor (TLR) pathway. We found that CAD patients with the variant (CC) genotype were associated with significantly higher levels of miR-146a. The targets of miR-146a, IRAK-1 and TRAF-6, were significantly lower in these patients (CC genotype). The lowest levels of NF- κ B and C-reactive protein (CRP) were found in patients with the homozygous C allele compared to the heterozygous GC and wildtype variants. The results of our study suggested a role for miR-146a as a target for lowering inflammation in CAD patients.^[10]

Inflammatory cytokines play an important role in the pathogenesis and development of CAD. Increased levels of proinflammatory cytokines have been associated with worse prognosis of CAD. Several cytokines, including interleukin (IL)-17 receptor β (IL-17R β), IL-23a, IL-2 β , IL-6, IL-2, IL-18R, and IL-21 are well known targets of miR-499.^[5, 11] MicroRNA-499 has been implicated in inflammatory diseases such as rheumatoid arthritis (RA), and pulmonary tuberculosis (PTB).^[5, 12]

A common miR-499 A/G SNP (rs3746444) located within the crucial seed sequence of miR-499 results in a change from an A:U pair to a G:U mismatch in the stem structure of miR-499.^[13, 14] The variant G allele has a less stable secondary structure than the wildtype allele and directly affects the

binding to target mRNAs, therefore altering protein expression and potentially contributing to disease susceptibility.

Coronary artery disease remains one of the leading causes of death worldwide, with an unusually high prevalence of early-onset CAD in Indians. South African Indians have a much higher prevalence of CAD compared to Black South Africans.^[15] To date, there is no literature available on rs3746444 in South African Indians. This study aimed to assess the frequency and role of this SNP in young Indian CAD patients compared to Indian and Black controls.

METHODS

Patient Recruitment and Sample Collection

Following institutional ethical approval (BE067/14), a total of 289 subjects were recruited into this study which included 105 CAD patients (diagnosed by angiography, mean age 37.5; range 24-45 years), 100 age-, sex-, and race-matched controls, and 84 age- and sex-matched Black controls. A full pathology report of clinical markers was assessed by routine laboratory testing at Global Clinical and Viral Laboratory (Durban, South Africa) - a fully accredited South African National Laboratory. The following parameters were tested: Haematology (Roche Sysmex 1800XT), Chemistry (Beckman Coulter DXC600), Endocrinology and high sensitivity C-reactive protein (hsCRP) (Siemens Centaur XP), Serology (BD Biosciences FACS Calibur), as per international standards to obtain levels of total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides, fasting glucose, 2-h glucose, fasting insulin, glycosylated haemoglobin (HbA1c), sodium, potassium, bicarbonate, chloride, urea, creatinine, and glomerular filtration rate. The physical measurements of weight, height, abdominal circumference, waist circumference and patient history were conducted by the cardiologist (Dr. S. Khan). The inclusion criteria for CAD patients were: Indian ancestry and unrelated, adults aged <45 years, and stable CAD confirmed at angiography. The exclusion criteria for controls included an acute coronary syndrome/revascularisation procedure in the preceding 3 months, chronic renal or liver disease, malignancy and known inflammatory or infectious disease.

DNA Extraction

Genomic DNA was extracted from the whole blood sample of each subject, according to the method described by Sambrook et al.^[16] Cells were transferred to 600 µl lysis buffer (0.5% sodium dodecyl sulphate (SDS), 150 mM NaCl, 10 mM ethylenediaminetetra-acetic acid (EDTA), 10 mM Tris-HCl (pH 8.0)). To this, RNase A (100 µg/ml; DNase-free) was added to the solution and incubated (37°C,

1 h). Proteinase K (200 µg/ml) was then added and incubated (3 h, 50°C). Protein contaminants were then precipitated by adding 5 mM 0.1% potassium acetate before centrifugation (5,000 ×g; 15 min). Supernatants containing genomic DNA were transferred to fresh tubes and extracted with 100% isopropanol on ice, and thereafter washed with 70% ethanol. DNA samples were dissolved in 10 mM Tris and 0.1 mM EDTA (pH 7.4, 4°C). DNA concentration was determined using the Nanodrop2000 spectrophotometer, and all samples were standardised to a concentration of 10 ng/µl.

Genotyping

An optimised polymerase chain reaction (PCR) was used to obtain the highest specificity and yield of the PCR product. This was achieved by amplification of the genomic DNA using 40 pmol of each primer (Forward Primer: 5'- ATTGGATGCCGCAGTGGT-3'; Reverse Primer: 5'- TGTTTAACTCCTCTCCACGTGATC-3').^[17] A no-template sample was run with the positive samples as a quality control measure against PCR contamination. The 25 µl reaction consisted of 200 µM of each dNTP, 2.5 mM MgCl₂, 1× Green GoTaq Flexi buffer, 0.5U Go-Taq DNA polymerase (Promega) and 30 ng genomic DNA template. PCR was performed under the following cycling conditions: 94°C for 5 min (initial denaturation), followed by 34 cycles of 94°C for 30 s, 59 °C for 30 s (annealing), 72°C for 30 s (extension), and 72°C for 7 min (final extension). PCR products were electrophoresed on agarose gel (1.8%, 0.5 mg/ml ethidium bromide) and visualised using the Uvitech image documentation system (Uvitech Alliance 2.7). PCR-RFLP was used to determine rs3746444 genotypes. 15 µl of each PCR product was subjected to restriction by 1 µl (10 u/µl) Bcl I and 2 µl 10× Buffer G (Fermentas). Overnight restriction occurred at 55°C, and thereafter restriction products were electrophoresed on agarose gel (3%, 0.5 mg/ml ethidium bromide) and visualised as was the PCR product. Two fragments of 239 bp and 24 bp were observed for the wild-type A allele, while the variant G allele yielded a single band of 263 bp. Restriction products were run alongside a DNA ladder for accurate reading of fragment sizes, thus enabling correct analysis of genotypes.

IL-6 enzyme-linked immunosorbent assay

A human IL-6 ELISA Kit II (BD OptEIA™) was used to measure the IL-6 levels of patients and controls (in duplicate). As per the user manual, enzyme-linked immunosorbent assay (ELISA) diluent (12ml of buffered protein base, 0.09% sodium azide) was added to a 96-well microtiter plate coated with antihuman IL-6 monoclonal antibody, and standards and samples were added into appropriate wells. Following incubation for 2 hr at room temperature, wells were rinsed with wash buffer (1× detergent solution with ProClin™ -150).

Working detector (containing streptavidin–horseradish peroxidase and biotinylated anti-human IL-6 monoclonal antibody) was added into each well and incubated for 1 hr at room temperature in the dark). Wells were washed as previously, seven times, followed by the addition of TMB One-Step Substrate Reagent (3,3',5,5'-tetramethylbenzidine) and incubated for 30 min at room temperature in the dark. Following incubation, stop solution (1M phosphoric acid) was added, and absorbance measured at 450 nm (reference, 570 nm) with a microplate reader (Bio-Tek μ Quant).

Statistical Analysis

The Hardy–Weinberg equilibrium was used to test for deviation of allele/genotype frequency. All other statistical analysis was performed with Graphpad prism software (version 5.0). Allele and genotype frequencies were calculated using the Fisher's exact and Chi square tests, respectively. Characteristics of CAD patients according to genotype was analysed by a non-parametric T-test. Results are expressed as mean \pm standard error. A p value less than 0.05 was considered statistically significant.

RESULTS

The miR-499 rs3746444 genotype distribution complied with the Hardy-Weinberg equilibrium in CAD patients, Indian controls, and Black controls (Chi-squared $p=0.143479$, $p=0.742181$, and $p=0.419581$ respectively). The variant (G) allele was found at a higher frequency in the total Indian group (34%) compared to the total Black population (22%) ($p=0.0070$, OR=1.796 95% CI 1.182 – 2.730; Table 1). Indian cases presented with higher frequency of the variant allele compared to Indian controls (38% vs. 29%, $p = 0.059$, respectively) (Table 5.1).

Table 5.1 miR-499 rs3746444 genotype and allele frequency in CAD patients and controls

Genotypes	CAD Patients (n=105)	Indian controls (n=100)	Total Indian Population (n=205)	Black controls (n=84)
AA	45 (43%)	52 (52%)	97 (47%)	49 (58.3%)
AG	40 (38%)	38 (38%)	78 (38%)	33 (39.3%)
GG	20 (19%)	10 (10%)	30 (15%)	2 (2.4%)
Allele				
A	130 (62%)	142 (71%)	272 (66%)	131 (78%)
G	80 (38%)	58 (29%)	138 (34%)	37 (22%)

Characteristics of study participants were reported previously.^[18] CAD patients' characteristics according to rs3746444 genotypes are presented in Table 5.2. There were no significant differences observed between the wild type and variant genotypes.

Table 5.2 Characteristics of CAD patients according to the miR-499 rs3746444 genotype

	miR-499 rs3746444 genotype		p-value
	Wild type (AA)	Variant (AG+GG)	
Total cholesterol (mmol/l)	5.36±0.25	5.34±0.25	ns
LDL (mmol/l)	3.36±0.26	3.44±0.22	ns
HDL (mmol/l)	0.92±0.04	0.93±0.03	ns
Triglycerides (mmol/l)	2.64±0.28	2.18±0.16	ns
BMI (kg/m ²)	28.17±0.81	28.21±0.51	ns
Fasting glucose (mmol/l)	6.82±0.52	5.99±0.27	ns
Fasting insulin (uIU/ml)	16.24±1.71	15.79±1.41	ns
HbA1c (%)	7.02±0.35	6.29±0.20	ns
hsCRP (mg/l)	6.73±0.87	8.92±1.91	ns
IL-6 (pg/ml)	2.18±0.57	3.02±0.77	ns

LDL = Low density lipoprotein, BMI = Body mass index, HbA1c = Glycated haemoglobin, hsCRP = High sensitivity C-reactive protein, ns = non-significant

Elevated hsCRP and IL-6 levels were observed in CAD patients harboring the variant genotypes compared to those with the wild type genotypes (8.92±1.91 vs. 6.73±0.87 mg/l, 3.02±0.77 vs. 2.18±0.57 pg/ml respectively) (Table 5.2, Figure 5.1).

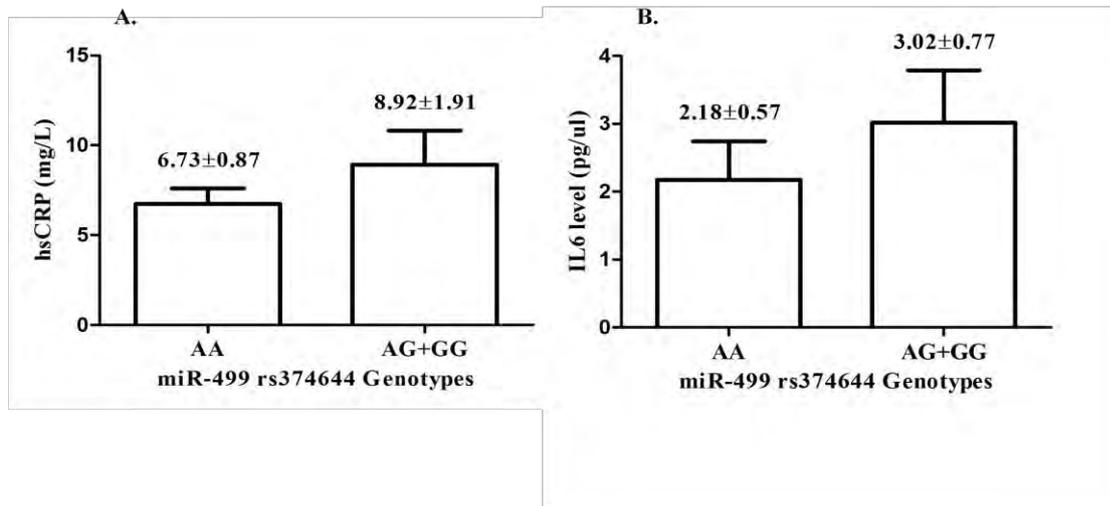


Figure 5.1 hsCRP and IL-6 levels of CAD patients with the miR-499 rs3746444 wildtype and variant genotypes

DISCUSSION

Single nucleotide polymorphisms in miR genes may alter a wide spectrum of biological processes by affecting the expression and/or target interaction of miRs,^[12]. A growing number of studies have revealed that rs3746444 affects the pathogenesis of several human diseases, including breast cancer,^[19] hepatocellular carcinoma,^[20] RA,^[5] CAD,^[21] chronic obstructive pulmonary disease,^[4] and TB,^[12].

The present study was the first investigation of the frequency of rs3746444 in young Indian CAD patients compared to Indian and Black controls. We found that the variant allele occurred at a higher frequency in the total Indian group compared to the total Black population. Indian cases presented with higher frequency of the variant allele compared to Indian controls. The highest mortality rates for CAD in South Africa occurs in Indians, followed by mixed race, white and black,^[15]. The higher frequency of the variant allele in our Indian study group may be a contributing factor to the higher risk profile for the development of CAD.

Yang *et al* observed that rs3746444 could affect the inflammatory response in RA patients,^[5]. C-reactive protein, erythrocyte sedimentation rate (ESR), IL-6, and TNF- α were used to indicate the degree of chronic inflammation *in vivo* in RA patients. The authors found that carriers of the heterozygous genotype had significantly higher levels of CRP and ESR compared to the homozygous wildtype and variant genotypes, indicating an important role for rs3746444 in the progress and inflammatory reaction of RA.

We observed elevated levels of hsCRP and IL-6 in CAD patients harboring the variant compared to those with the wild type genotypes, but these were not significant. MiR-499 is known to target several inflammatory cytokines, including IL-6, which directly regulates the production of CRP,^[11]. The change from A-to-G in miR-499 rs3746444 may interfere with its binding to and interaction with targets such as IL-6. Thus miR-499 can affect the synthesis of CRP and regulate inflammation in CAD patients. Li *et al* observed no link between rs3746444 and PTB risk in a Chinese Han group, but found that Tibetans carrying the variant allele exhibited increased risk of PTB,^[12].

Previous studies revealed that the GG and AG genotypes of rs3746444 conferred increased risk for CAD,^[21] and ischemic stroke,^[22], respectively. To date, there have been only two published studies on the miR-499 rs3746444 with regards to CAD,^[17, 21]. Recently, Zhi *et al* found that the GG genotype was associated with a 3.23-fold increased risk of CAD,^[21]. In contrast, Xiong *et al* found no association between rs3746444 and CAD in a Chinese Han population,^[17].

Our findings suggest that miR-499 may be a target for regulating inflammation in CAD patients. A larger follow up study investigating the influence of this miR-499 SNP may provide additional clarification.

CONCLUSION

The precise mechanisms regulating miR expression and target interaction remain unknown, but SNPs are known to play an important role. Small variation in miRs may affect several targets and result in diverse functional consequences, therefore SNPs in miRs may represent ideal candidate biomarkers for diseases prognosis and serve as a useful tool in therapeutic advances.

FUNDING

National Research Foundation and College of Health Science Scholarships.

CONTRIBUTORS

All authors have contributed to the manuscript and have seen and approved the final version.

COMPETING INTERESTS

None

PATIENT CONSENT

Obtained

ETHICS APPROVAL

Ethics approval was obtained.

KEY MESSAGES

- South African Indians have a higher prevalence of early-onset CAD compared to Black South Africans.
- Single nucleotide polymorphisms in microRNA genes, such as miR-499 rs3746444 may affect the expression and/or target interaction.
- microRNAs are potentially useful as prognostic or therapeutic biomarkers in CAD.

REFERENCES

1. Andreassi M, Botto N, Cocci F, et al. Methylenetetrahydrofolate reductase gene C677T polymorphism, homocysteine, vitamin B12, and DNA damage in coronary artery disease. *Hum Genet.* 2003;112(2):171-177.
2. Wang JX, Jiao JQ, Li Q, et al. miR-499 regulates mitochondrial dynamics by targeting calcineurin and dynamin-related protein-1. *Nat Med.* 2011;17.
3. Corsten MF, Dennert R, Jochems S, et al. Circulating MicroRNA-208b and MicroRNA-499 Reflect Myocardial Damage in Cardiovascular Disease. *Circ: Cardiovasc Genet.* 2010;3(6):499-506.
4. Li LJ, Gao LB, Lv ML, et al. Association between SNPs in pre-miRNA and risk of chronic obstructive pulmonary disease. *Clin Biochem.* 2011;44:813-816.
5. Yang B, Chen J, Li Y, et al. Association of polymorphisms in pre-miRNA with inflammatory biomarkers in rheumatoid arthritis in the Chinese Han population. *Hum Immunol.* 2012;73:101-106.
6. Zhou B, Rao L, Peng Y, et al. Common genetic polymorphisms in pre-microRNAs were associated with increased risk of dilated cardiomyopathy. *Clin Chim Acta.* 2010;411(17-18):1287-1290.
7. Li L, Sheng Y, Lv L, et al. The Association between Two MicroRNA Variants (miR-499, miR-149) and Gastrointestinal Cancer Risk: A Meta-Analysis. *PLoS ONE.* 2013;8(11):e81967.
8. Qiu M-T, Hu J-W, Ding X-X, et al. Hsa-miR-499 rs3746444 Polymorphism Contributes to Cancer Risk: A Meta-Analysis of 12 Studies. *PLoS ONE.* 2012;7(12):e50887.
9. Ono K, Kuwabara Y, Han J. MicroRNAs and cardiovascular diseases. *FEBS J.* 2011;278:1619-1633.
10. Ramkaran P, Khan S, Phulukdaree A, et al. miR-146a Polymorphism Influences Levels of miR-146a, IRAK-1, and TRAF-6 in Young Patients with Coronary Artery Disease. *Cell Biochem Biophys.* 2013:1-8.
11. Hashemi M, Eskandari-Nasab E, Zakeri Z, et al. Association of pre-miRNA-146a rs2910164 and premiRNA-499 rs3746444 polymorphisms and susceptibility to rheumatoid arthritis. *Mol Med Rep.* 2013;7:287-291.
12. Li D, Wang T, Song X, et al. Genetic study of two single nucleotide polymorphisms within corresponding microRNAs and susceptibility to tuberculosis in a Chinese Tibetan and Han population. *Hum Immunol.* 2011;72(7):598-602.

13. Wang N, Tian ZQ, Li Y, et al. An A/G polymorphism rs3746444 in miR-499 is associated with increased cancer risk: a meta-analysis. *Genetics and Molecular Research*. 2013;12(3):3955-3964.
14. Qi J-H, Wang J, Chen J, et al. High-resolution melting analysis reveals genetic polymorphisms in MicroRNAs confer hepatocellular carcinoma risk in Chinese patients. *BMC Cancer*. 2014;14(1):643.
15. Revised Burden of Disease Estimates for the comparative risk factor assessment, South Africa 2000. Cape Town: Medical Research Council 2006. [<http://www.mrc.ac.za/bod/RevisedBurdenofDiseaseEstimates1.pdf>]. 2006.
16. Sambrook J, Russell DW. Rapid isolation of mammalian DNA in *Molecular Cloning: A laboratory manual*. 3 ed. New York: COLD SPRING HARBOR LABORATORY PRESS.; 2001.
17. Xiong XD, Cho M, Cai XP, et al. A common variant in pre-miR-146 is associated with coronary artery disease risk and its mature miRNA expression. *Mut Res*. 2014;761(0):15-20.
18. Phulukdaree A, Khan S, Moodley D, et al. GST polymorphisms and early-onset coronary artery disease in young South African Indians. *S Afr Med J*. 2012;102(7):627-630.
19. Catucci I, Yang R, Verderio P, et al. Evaluation of SNPs in miR-146a, miR196a2 and miR-499 as low-penetrance alleles in German and Italian familial breast cancer cases. *Hum Mut*. 2010;31(1):E1052-E1057.
20. Zou HZ, Zhao YQ. Positive association between miR-499A>G and hepatocellular carcinoma risk in a Chinese population. *Asian Pacific Journal of Cancer Prevention*. 2013;14(3):1769-1772.
21. Zhi H, Wang L, Ma G, et al. Polymorphisms of miRNAs genes are associated with the risk and prognosis of coronary artery disease. *Clin Res Cardiol*. 2012;101:289-296.
22. Liu Y, Ma Y, Zhang B, et al. Genetic polymorphisms in pre-microRNAs and risk of ischemic stroke in a Chinese population. *J Mol Neurosci*. 2014;52(473-480).

CHAPTER 6

miR-146a Polymorphism Influences Levels of miR-146a, IRAK-1, and TRAF-6 in Young Patients with Coronary Artery Disease

Prithiksha Ramkaran¹, Sajidah Khan², Alisa Phulukdaree¹, Devapregasan Moodley¹, Anil A Chuturgoon^{1*}

¹Discipline of Medical Biochemistry, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa

A Phulukdaree, PhD

D Moodley, PhD

A A. Chuturgoon, PhD

²Department of Cardiology, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa

S Khan, MB ChB, FCP(SA), PhD

*Corresponding author - Telephone: +27 31 260 4404. E-mail: chutur@ukzn.ac.za

Cell Biochemistry and Biophysics (2013), 68(2), 259-266

DOI 10.1007/s12013-013-9704-7

ABSTRACT

Modulation of nuclear factor KappaB (NF- κ B) activation may play a role in regulating inflammatory conditions associated with coronary artery disease (CAD). MicroRNA-146a (miR-146a) primarily targets interleukin-1 receptor-associated kinase 1 (IRAK-1) and tumor necrosis factor (TNF) receptor associated factor 6 (TRAF-6), which results in inhibition of NF- κ B via the TLR pathway. This study investigated the influence of the miR-146a GC rs2910164 on miR-146a expression in young South African Indians with CAD. CAD patients and controls were genotyped by PCR-RFLP and miRNA-146a levels were measured by qPCR. IRAK-1, TRAF-6, and NF- κ B expression was determined by western blot. No differences in genotypic frequency was found (GG: 45% vs. 47%, GC: 46% vs. 41%, CC: 9% vs. 12%) in controls and patients respectively (odds ratio (OR) = 1.025; 95% confidence interval (CI) 0.6782 – 1.550; $p=0.9164$). Significantly higher levels of miR-146a was associated with CAD patients with the CC genotype (6.25-fold increase relative to controls and patients with the wildtype variant, $p<0.0001$). Significantly lower levels of IRAK-1 (0.38 ± 0.02 ; $p=0.0072$) and TRAF-6 (0.44 ± 0.02 ; $p=0.0146$) was found in CAD patients with the CC genotype. The lowest levels of NF- κ B and C-reactive protein (CRP) were found in patients with the homozygous C allele compared to the heterozygous GC and wildtype variants. We propose a role for miR-146a in TLR signalling through a negative feedback mechanism involving the attenuation of NF- κ B by down-regulation of IRAK-1 and TRAF-6. Our observations implicate miR-146a as a target for lowering inflammation in CAD patients.

Key Words

miRNA-146a, IRAK-1, TRAF-6, NF- κ B, C-reactive protein

1. Introduction

Micro-RNAs (miRs) are a class of small non-coding RNA that negatively regulate the expression of cognate target genes [1, 2]. MiRs attach to the 3'-untranslated region (3'-UTR) of their target messenger RNAs (mRNA) through their highly conserved seed sequence (2-7 nucleotides) [3, 4]. A single miR can potentially target hundreds of mRNA transcripts and can thus modulate transcriptional programs that affect entire cellular networks [5, 6]. Genetic alterations in miR sequences affect precursor processivity and thus maturation and expression of miRs. In addition, changes in the seed sequence alter binding kinetics between the mature miR and 3'-UTR target. It is conceivable that since miRs regulate magnitude of targets, small changes in genetic sequence will have large implications for transcriptional regulation and ultimately for the development of disease [7]. Single nucleotide polymorphisms (SNPs) have been well investigated in exons [8, 9], but little is known about their effect in miR and role in disease conditions. The miR-146a rs2910164 has been widely investigated in rheumatoid arthritis, tuberculosis and several cancers [1, 7, 10].

Single nucleotide polymorphisms are the most common type of genetic variation in the human genome and have been well investigated in protein coding genes which affect the functioning of proteins and consequently render susceptibility to disease [8, 9]. There are limited studies focusing on the relationship between miR-146a expression and cardiovascular diseases [2, 11-13]. miR-146a is involved in regulating the innate immune, and inflammatory response pathways [14]. A common G/C SNP (rs2910164) located within the crucial seed sequence of miR-146a [15] causes a change from a G:U pair to a mismatched C:U in the stem region and was shown to affect the expression of mature miR-146a [1, 9]. Whether genetic variants of miR-146a confer differences in expression of miR-146a in CAD is unclear [1, 16, 17]. If such differences do occur, whether they are enough to produce significantly different regulatory effects on major mRNA targets remains unknown.

miR-146a primarily targets IRAK-1 and TRAF-6, which are involved in TLR and IL-1 receptor (IL-1R) signalling [18]. Upon stimulation of the TLR, the adaptor proteins myeloid differentiation primary-response gene 88 (MyD88) and TLR domain-containing adaptor-inducing interferon- β (TRIF) are recruited from the cell cytoplasm to the receptor, resulting in the activation of two independent branches of TLR signalling [19]. In the MyD88-dependent branch, MyD88 recruits IRAK-1, which in turn phosphorylates and activates TRAF-6. This chain of events leads to the phosphorylation and degradation of inhibitory kappa B ($\text{I}\kappa\text{B}$), allowing NF- κB to enter the cell nucleus and activate the transcription of inflammatory genes [7, 19]. The effect of dysregulated miR-146a expression on its target molecules IRAK-1 and TRAF-6 and their downstream functions in the TLR signalling pathway remain unclear. It is likely that alterations in miR-146a expression caused by genetic variation in miR-146a such as SNPs may affect TLR signalling.

CAD has been the leading cause of fatality worldwide since 1990 and this trend is expected to continue in the future [20, 21]. At present, developing countries such as South Africa and India contribute a large share to the global burden of CAD and are expected to account for 85% by 2020 [20, 22]. Most South African Indians are descendants of indentured labourers who were brought from India between 1860 and 1911 [23]. Indians in India and South Africa show early onset of CAD, one to two decades earlier than other ethnic groups [24], indicating a genetic link. The highest death rates for CAD in South Africa occurs in Indians [25].

The present study aimed to investigate the influence of miR-146a rs2910164 on miR-146a expression in young South African Indian CAD patients. This is the first investigation of the miR-146a SNP in this population group

2. Methods

2.1 Patient recruitment and Sample collection

A total of 106 young Indian male CAD patients and 100 age- race- and sex-matched controls were enrolled following institutional ethical approval (BE154/010). A full pathology report of clinical markers was assessed by routine laboratory testing at the South African National Accredited System Global Clinical and Viral Laboratory (Amanzimtoti, South Africa). The following parameters were tested: Haematology (Roche Sysmex 1800XT), Chemistry (Beckman Coulter DXC600), Endocrinology and hsCRP (Siemens Centaur XP), Serology (BD Biosciences FACS Calibur) as per international standards to obtain levels of total cholesterol, HDL, LDL, triglycerides, fasting glucose, 2h glucose, fasting insulin, glycosylated haemoglobin, sodium, potassium, bicarbonate, chloride, urea, creatinine, glomerular filtration rate, cell death (CD) 4 count, CD8 count, CD45 count and CD3 count. The physical measurements of weight, height, abdominal circumference, waist circumference and patient history were conducted by the clinician. The inclusion criteria for CAD patients were: Indian ancestry and unrelated, adults aged <45 years, and stable CAD confirmed at angiography. The exclusion criteria for controls included an acute coronary syndrome/ revascularisation procedure in the preceding 3 months, chronic renal or liver disease, malignancy and known inflammatory or infectious disease.

2.2 DNA extraction

Genomic DNA was extracted from the whole blood sample of each patient and control according to the method described by Sambrook et al. 2001 [26]. Cells were transferred to 600µl lysis buffer (0.5% sodium dodecyl sulphate (SDS), 150mM NaCl, 10mM ethylenediaminetetra-acetic acid (EDTA), 10mM Tris-HCl (pH 8.0)). To this, RNase A (100 µg/ml; DNase-free) was added to the solution and incubated (37°C, 1 h). Proteinase K (200 µg/ml) was then added and incubated (3 h, 50°C). Protein contaminants were then precipitated by adding 5mM 0.1% potassium acetate before centrifugation (5000 xg; 15 min). Supernatants containing genomic DNA were transferred to fresh tubes and extracted with 100% isopropanol on ice and thereafter washed with 70% ethanol. DNA samples were dissolved in 10mM Tris and 0.1mM EDTA (pH 7.4, 4°C). DNA concentration was determined using the Nanodrop2000 spectrophotometer, and all samples were standardised to a concentration of 10ng/µl.

2.3 Genotyping

An optimised polymerase chain reaction (PCR) was used to obtain the highest specificity and yield of the 147bp PCR product. This was achieved by amplification of the genomic DNA using 40 pmol of each primer (Forward Primer: 5'-CATGGGTTGTGTCAGTGTCAGAGCT-3' ; Reverse Primer: 5'-TGCCTTCTGTCTCCAGTCTTCCAA -3') [16]. A no-template sample was run with the positive samples as a quality control measure against PCR contamination.

The 30µl reaction consisted of 200µM of each dNTP, 2.5mM MgCl₂, 1× Green GoTaq Flexi buffer, 0.2U GoTaq DNA polymerase (Promega) and 30ng genomic DNA template. PCR was performed under the following cycling conditions: 94°C for 10 minutes (initial denaturation), followed by 30 cycles of 94°C for 30 seconds, 65°C for 30 seconds (annealing), and 72°C for 7 minutes (final extension). PCR products were electrophoresed on agarose gel (1.8%, 0.5 mg/ml ethidium bromide) and visualised using the Uvitech image documentation system (Uvitech Alliance 2.7).

PCR-RFLP was used to determine the miR-146a rs2910164 genotypes. 15µl of each PCR product was subjected to restriction by 1.5 µl (10u/µl) *Sac* I and 2µl 10× Buffer-*Sac* I (Fermentas). Overnight restriction occurred at 37°C and thereafter restriction products were electrophoresed on agarose gel (3%, 0.5 mg/ml ethidium bromide) and visualised as was the PCR product. Presence of the wild-type G-allele resulted in no cleavage of the PCR product. The variant C-allele yielded two fragments of 122 and 25bp. The homozygous genotype yielded three bands of 147, 122, and 25bp. Restriction products were run alongside a DNA ladder for accurate reading of fragment sizes, thus enabling correct analysis of genotypes.

2.4 RNA extraction

Total RNA was isolated from peripheral blood mononuclear cells (PBMC) from each miR-146a rs2910164 genotype in CAD patients and controls. RNA was isolated with Trizol reagent following an in-house protocol. Briefly, Trizol (300 μ l) was added to PBMCs and frozen for 1 hour at -80°C. Thereafter, chloroform (100 μ l) was added and centrifuged at (12000 \times g, 15 minutes, 4°C). Isopropanol (250 μ l) was added to the aqueous phase and once again subjected to freezing (1 hour, -80°C). This was followed by centrifuging (12000 \times g, 20 minutes, 4°C), and then washing with 500 μ l cold ethanol (75%). Finally, samples were centrifuged at 7400 \times g for 15 minutes (4°C). Ethanol was removed and RNA pellets were resuspended in 15 μ l nuclease-free water.

RNA was quantified using the Nanodrop2000 spectrophotometer. The ratio of absorbance at 260nm and 280nm was used to assess the purity of RNA, and samples were run on 2% agarose gel to check the integrity of the RNA. All RNA samples were standardised to a concentration of 100ng/ μ l.

Total RNA was used to prepare cDNA using the miScript II RT kit (miScript Reverse Transcriptase Mix, 10 \times miScript Nucleics Mix, 5 \times miScript HiSpec Buffer; Qiagen, Catalog no. 218161) according to the manufacturer's instructions.

2.5 Quantitative PCR

miR-146a expression was analysed using the miScript SYBR Green PCR kit (Qiagen, Catalog no. 218073) according to the manufacturer's instructions. The miR-146a expression assay was run in triplicates. Human U6 was used as the internal control.

The comparative threshold cycle (C_t) method was used to assess the relative changes in A no-template control was run with the positive samples to assess the overall specificity of the reaction [27]. expression. $2^{-\Delta\Delta C_t}$ represents the fold change in miR-146a expression between sample groups.

2.6 Western Blot Analysis

Cytobuster™ reagent (Novagen) supplemented with protease and phosphatase inhibitors (Roche, 05892791001 and 04906837001 respectively) was used for protein isolation. Cytobuster (200 μ l) was added to PBMCs (4°C, 10min) and centrifuged (180 x g; 4°C, 10min) to obtain a crude protein extract. Protein samples were quantified using the bicinchoninic acid assay and standardized to 0.5mg/ml. Protein sample were prepared with laemlii buffer (0.5M Tris (pH 6.8), glycerol, 10% sodium dodecyl sulfide (SDS), 0.5% bromophenol blue, β -mercaptoethanol) and denatured at 95°C.

Samples (25µl) were electrophoresed on 7.5% SDS-polyacrylamide gel electrophoresis gels and thereafter transferred to nitrocellulose membranes using the Trans-Blot® Turbo™ Transfer system (BIORAD). Membranes were blocked with 5% bovine serum albumin (BSA) in Tris buffer saline (20mM Tris-HCl (pH 7.4), 500mM NaCl and 0.01% Tween 20 (TBST)) for 1 hour, and incubated with primary antibody (IRAK-1; Cell Signalling Technology 4769, TRAF-6; Cell Signalling Technology 8347, NF-κB; ab17742, and β-actin; ab8226, 1:1000) in 1% BSA in TBST at 4°C overnight. Membranes were then washed (TBST, 4× 15min) and treated with secondary antibody (Anti-rabbit IgG, HRP-linked; Cell Signalling Technology 4769, 1:2000) for 1 hour at room temperature. Membranes were washed again (TBST, 4× 15min) and protein expression was detected by the LumiGLO® chemiluminescent substrate system (KPL) with the Alliance 2.7 Image Documentation System (UViTech). Protein bands were standardised against β-actin, and analyzed with the Advanced Image Analysis software (UViTech v12.14). The results were expressed as mean relative band intensity (RBI).

2.7 Statistical Analysis

The Hardy-Weinberg equilibrium was used to test for deviation of allele/genotype frequency. All other statistical analysis was performed with Graphpad prism software (version 5.0). Allele and genotype frequencies were calculated using the Fisher's exact and Chi-square tests respectively. The comparison of miR-146a expression between CAD patients and controls were done by a nonparametric t-test. All other calculations were done by performing a 1way ANOVA.

Results are expressed as mean ± standard error. A p value less than 0.05 was considered statistically significant.

3. Results

Amongst the clinical parameters assessed between the groups, body mass index (BMI), hypertension, diabetes, triglycerides, fasting glucose and HbA1c was higher in patients than controls (Table 6.1, published data).

Table 6.1 Demographics and clinical parameters

	Control (n=100)	Cases (106)	p value
Prediabetic/Diabetic (n (%))	39 (39)	59(58)	1.000
Age (years)	37.5 (0.44)	37.6 (0.40)	0.856
BMI (kg/m ²)	26.5 (0.47)	28.2 (0.46)	0.011*
Laboratory parameters			
Total Cholesterol (mmol/L)	5.48 (0.10)	5.36 (0.18)	0.583
LDL (mmol/L)	3.72 (0.09)	3.42 (0.17)	0.115
HDL (mmol/L)	0.96 (0.03)	0.93 (0.03)	0.358
Triglycerides (mmol/L)	1.88 (0.18)	2.37 (0.15)	0.0399*
Fasting Glucose (mmol/L)	5.45 (0.16)	6.34 (0.27)	0.0054*
Fasting Insulin (uIU/ml)	16.34 (1.26)	16.02 (1.08)	0.850
HbA1c (%)	5.70 (0.10)	6.61 (0.19)	<0.0001*
hsCRP (mg/L)	6.69 (1.12)	8.05 (1.15)	0.400
Medication			
	(%)	(%)	
Statins	3	84	
Aspirin	0	87	
Beta blockers	0	79	
Nitrates	0	37	
ACE inhibitors	2	72	

*Clinical parameters represented as mean±standard error of the mean. *p<0.01*

The genotype distribution complied with the Hardy-Weinberg equilibrium in patients and controls (Chi-square $p=0.7356$ and $p=0.8501$ respectively). No significant difference was observed in distribution of the miR-146a genotypes between the patients and controls ($p=0.9164$, OR=1.025, 95% CI 0.6782 – 1.550; Table 6.2). The rare C allele was more frequent in CAD patients (69) than controls (64).

Table 6.2 miR-146a rs2910164 G/C genotype and allele frequency distribution in CAD patients and controls

Frequency n (%)	CAD patients	Controls	p-value
Genotype			*0.6320
GG	50 (47)	45 (45)	
GC	43 (41)	46 (46)	
CC	13 (12)	9 (9)	
Allele			**0.9164
G	143 (67)	136 (68)	
C	69 (33)	64 (32)	

*Chi-squared test $X^2=0.9176$, 2 DF

**Fisher's exact test $p=0.9164$, RR=1.013 (95% CI 1.8181 – 1.254), OR=1.025 (95% CI 0.6782 – 1.550)

RR: Relative risk, CI: Confidence interval, OR: Odds ratio, DF: degrees of freedom

Relative to the controls, CAD patients exhibited a 2.58 fold increase in miRNA-146a expression ($p=0.3939$) (Figure 6.1). CAD patients with the miR-146a rs2910164 CC genotype expressed significantly higher levels of miR-146a (6.25 fold increase compared to controls and CAD patients with the reference GG genotype) *** $p<0.0001$ (Figure 6.2).

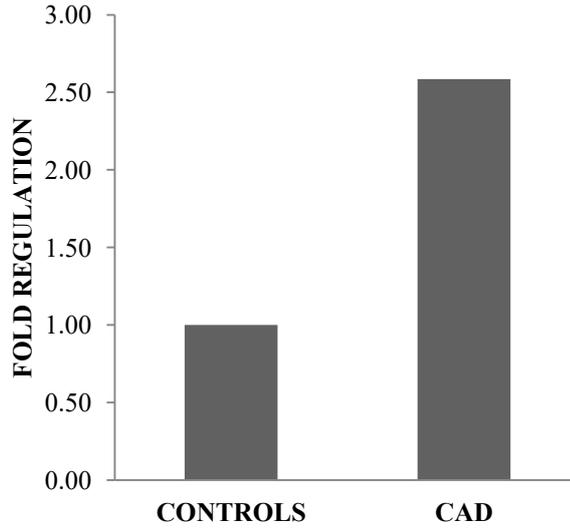


Figure 6.1 Fold change in miR-146a expression levels in CAD patients relative to controls

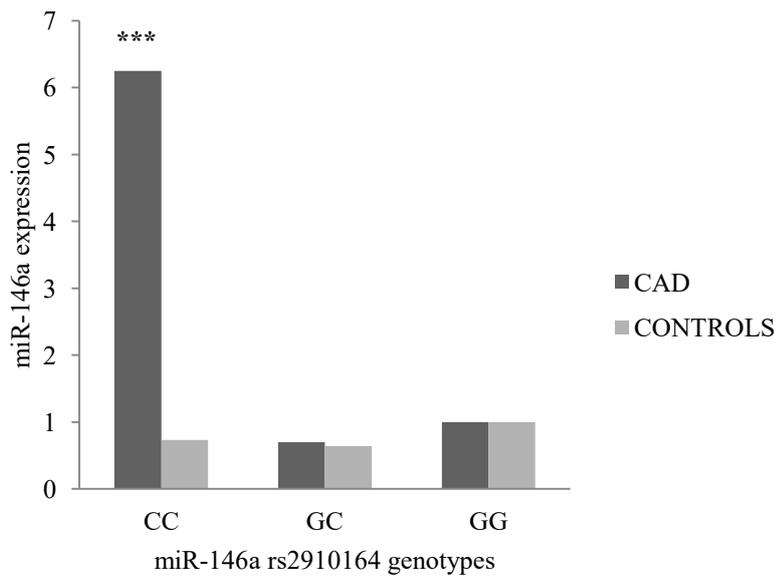


Figure 6.2 miR-146a expression in CAD patients and controls with different genotypes *** $p < 0.0001$

Patients with the CC genotype expressed significantly lower levels of IRAK-1 (0.38 ± 0.02 ; $p = 0.0072$; compared to GC, Figure 6.3) and TRAF-6 (0.44 ± 0.02 ; $p = 0.0146$; compared to GC and GG, Figure 6.4). These patients also expressed the lowest levels of NF- κ B compared to the other genotypes, although this did not reach statistical significance (7.18 ± 0.32 ; $p = 0.4843$, Figure 6.5).

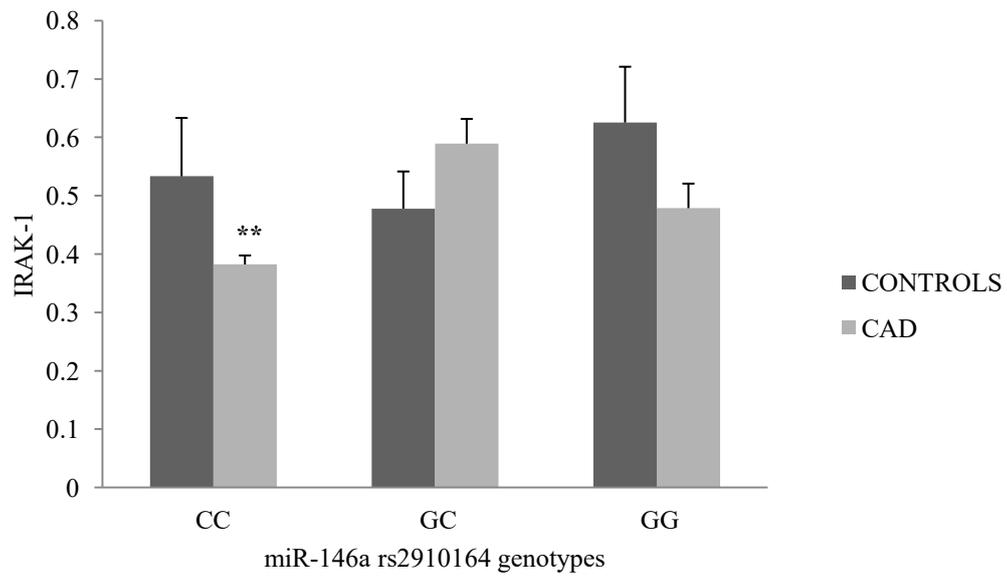


Figure 6.3 IRAK-1 expression in CAD patients (**p=0.0072) and controls

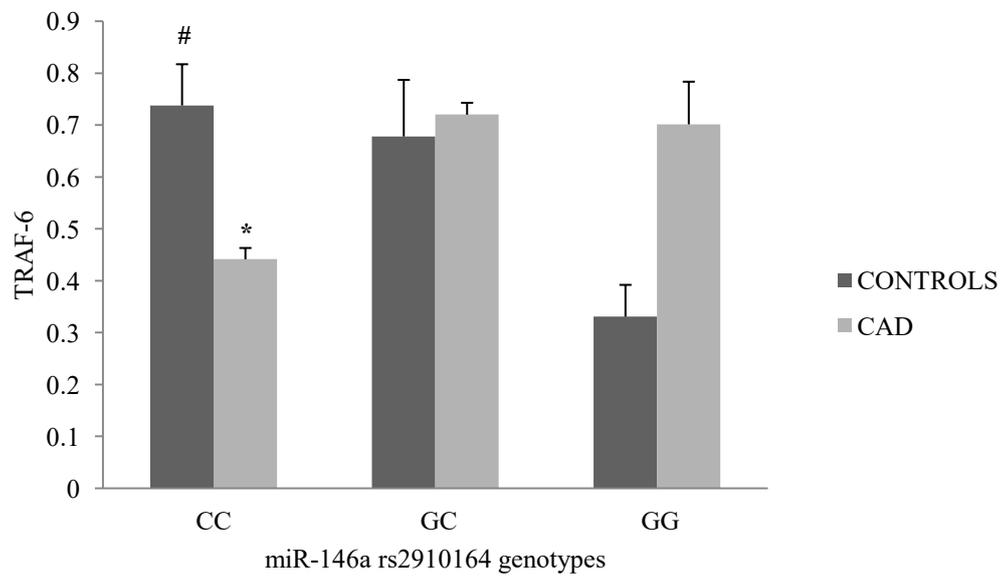


Figure 6.4 TRAF-6 expression in CAD patients (*p=0.0146) and controls (#p=0.0247)

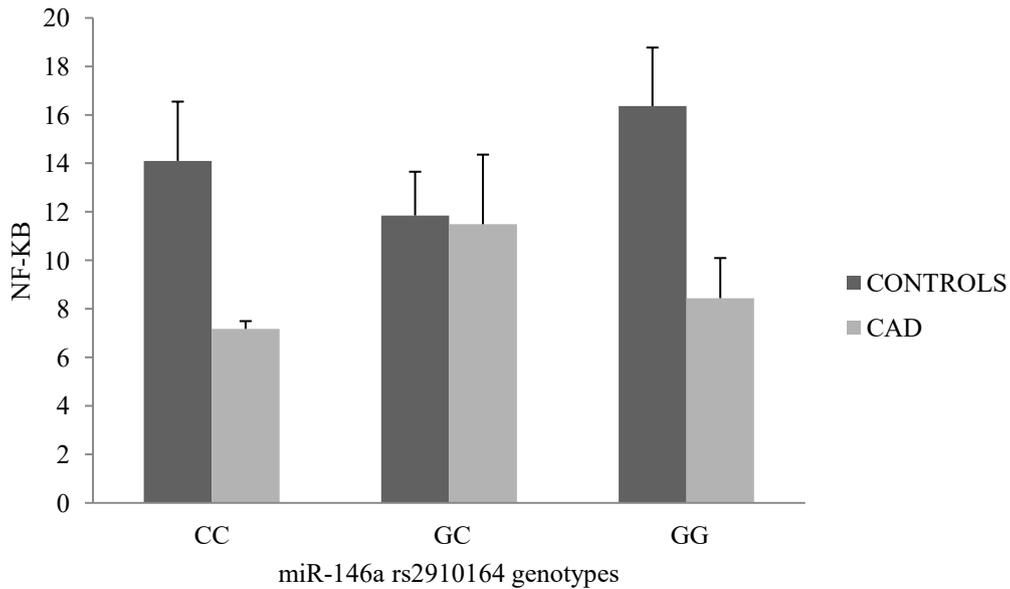


Figure 6.5 NF-κB expression in CAD patients and controls

CAD patients and controls with the CC genotype presented with the lowest mean hsCRP levels compared to the other genotypes (6.53 ± 1.47 and 4.23 ± 1.17 mg/L respectively). However this did not reach statistical significance ($p=0.7989$ for CAD patients and $p=0.9683$ for controls, Table 6.3).

Table 6.3 Mean hsCRP levels (mg/L) between genotypes in CAD patients and controls

	CAD patients	Controls
Genotype	Mean hsCRP levels (\pm standard deviation)	
GG	7.49 ± 1.77	7.67 ± 2.22
GC	9.01 ± 1.88	6.17 ± 1.04
CC	6.53 ± 1.47	4.23 ± 1.17
p-value	0.7989	0.9683

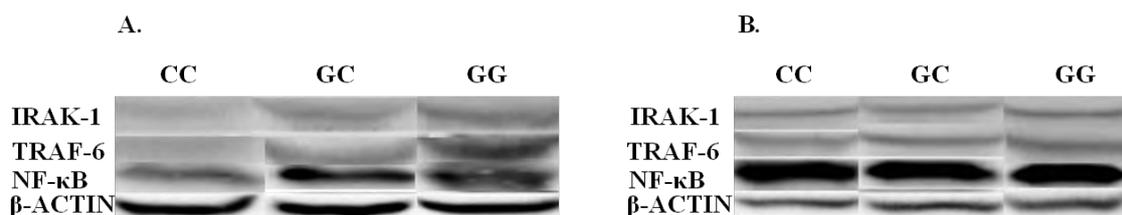


Figure 6.6 The expression of IRAK-, TRAF-6, and NF-κB in **(A)** CAD patients compared to **(B)** Controls.

4. Discussion

The present study investigated the possible association between the miR-146a rs2910164 and miR-146a expression in young South African Indian CAD patients.

CAD patients with the miR-146a rs2910164 CC genotype expressed significantly higher levels of miR-146a (6.25 fold increase compared to controls and CAD patients with the reference GG genotype; $p < 0.0001$) and significantly lower levels of IRAK-1 (0.38 ± 0.04 ; $p = 0.0072$; compared to GC) and TRAF-6 (0.44 ± 0.05 ; $p = 0.0146$; compared to GC and GG). Furthermore, these patients also expressed the lowest levels of NF-κB and hsCRP compared to the other genotypes, although this did not reach statistical significance. We propose a possible mechanism for the observed phenomenon.

IRAK-1 and TRAF-6 are both involved in the MyD88-dependent branch of the TIR signalling pathway [19, 28]. MyD88 is important for the activation of inflammatory cytokines triggered by all TIRs. Upon stimulation, MyD88 recruits IRAK-1 to the receptor. IRAK-1 is phosphorylated and complexed with TRAF-6, and together they dissociate from the receptor. A series of further interactions lead to phosphorylation of IκB, thereby releasing the transcription factor NF-κB into the nucleus and allowing the induction of inflammatory genes [28].

IRAK-1 and TRAF-6 are known targets of miR-146a [18]. The promoter region of miR-146a contains several binding sites for the transcription factor NF-κB, and the induced expression of miR-146a occurs in a NF-κB-dependent manner [7].

miR-146a inhibits IRAK-1 and TRAF-6 expression through negative feedback regulation (Figure 7) [7, 29]. Therefore, higher levels of miR-146a levels lead to increased inhibition of IRAK-1 and TRAF-6, causing decreased levels of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, TNF-α [29]. These cytokines are known regulators of CRP [30], and a decrease in their levels consequently reduce CRP levels.

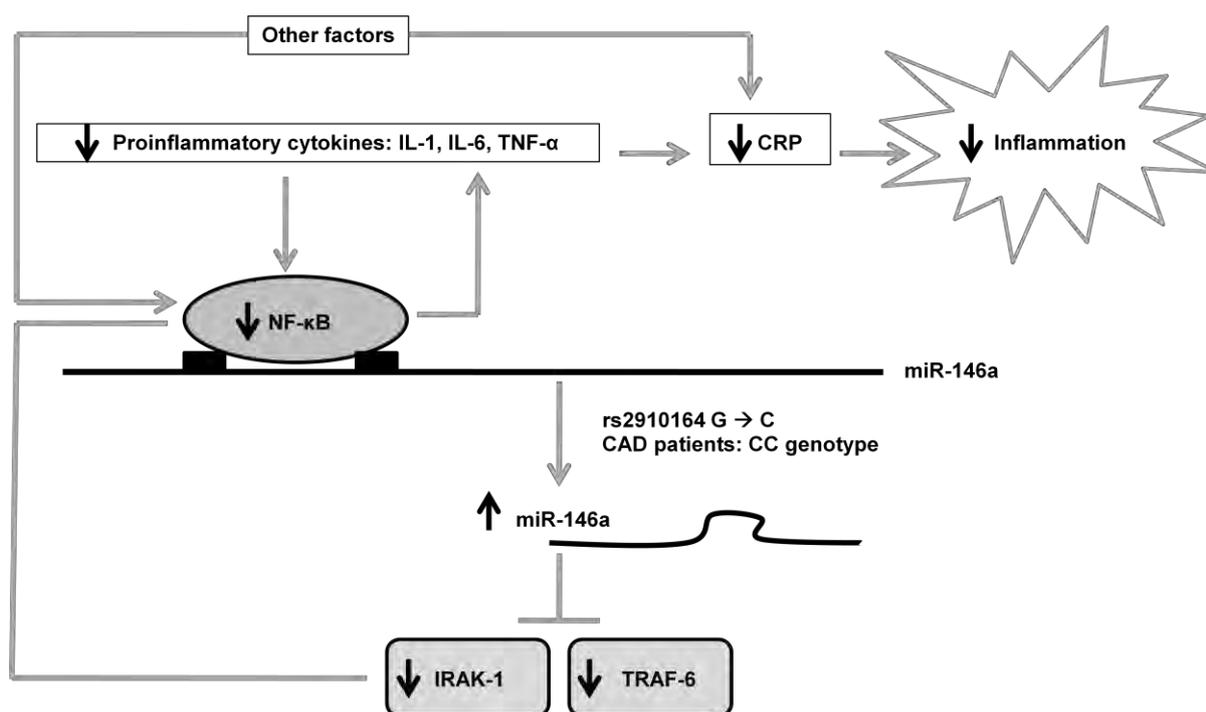


Figure 6.7 The role of miRNA-146a in the inflammatory response through a negative feedback mechanism (Author's own work)

Decreased hsCRP levels observed in CAD patients with the CC genotype may be as a result of them expressing significantly higher levels of miR-146a, increased binding to and inhibition of IRAK-1 and TRAF-6.

In contrast to our study, a previous investigation of the miR-146a rs2910164 in hepatocellular carcinoma by Xu et al. observed an increased production of miR-146a associated with the GG genotype [9]. In 2008, Shen et al. observed an increase in miR-146a expression in breast/ovarian cancer patients with the variant C allele [31]. However, neither of these studies investigated the target molecules of miR-146a and downstream effects of dysregulated miR-146a. This is the first study to investigate the miR-146a rs2910164 and its influence on miR-146a and miR-146a target molecule expression in young South African Indian CAD patients.

Since high levels of pro-inflammatory cytokines and CRP contribute to the chronic inflammation seen in CAD, the CC genotype serves a protective function by increasing miR-146a levels and reducing inflammation. Our study suggests miR-146a as an important modulator of the inflammatory response. Based on these findings, miR-146a could serve as a potential target in therapeutic interventions towards lowering inflammation and alleviating the complications in CAD.

Study limitations include sample size, lack of exclusion criteria on BMI, hypertension, diabetes, and effects of medication. The involvement of additional SNPs and/or regulatory molecules which are yet to be discovered may be a confounding factor in our study. A follow up study with larger patient populations would provide further clarification.

Funding

This work was supported by the National Research Foundation and College of Health Science Scholarships.

Conflict of Interest

None declared.

References

1. Hung, P.S., et al., *Association between the rs2910164 polymorphism in pre-mir-146a and oral carcinoma progression*. *Oral Oncology*, 2012. **48**: p. 404-408.
2. Takahashi, Y., et al., *Expression of miR-146a/b is associated with the Toll-like receptor 4 signal in coronary artery disease: effect of renin-angiotensin system blockade and statins on miRNA-146a/b and Toll-like receptor 4 levels*. *Clinical Science*, 2010. **119**: p. 395-405.
3. Zorc, M., et al., *Catalog of MicroRNA Seed Polymorphisms in Vertebrates*. *PLoS ONE*, 2012. **7**(1): p. e30737.
4. Ellwanger, D.C., et al., *The sufficient minimal set of miRNA seed types*. *Bioinformatics*, 2011. **27**(10): p. 1346-1350.
5. Hu, Z., et al., *Common genetic variants in pre-microRNAs were associated with increased risk of breast cancer in Chinese women*. *Human Mutation*, 2008. **30**(1): p. 79-84.
6. Mittal, R.D., et al., *Investigative role of pre-microRNAs in bladder cancer patients: a case-control study in North India*. *DNA AND CELL BIOLOGY*, 2011. **30**(6): p. 401-406.
7. Li, L., X. Chen, and Y. Li, *MicroRNA-146a and human disease*. *Scandinavian Journal of Immunology*, 2010. **71**: p. 227-231.
8. Yang, B., et al., *Association of polymorphisms in pre-miRNA with inflammatory biomarkers in rheumatoid arthritis in the Chinese Han population*. *Human Immunology*, 2012. **73**: p. 101-106.
9. Xu, T., et al., *A functional polymorphism in the miR-146a gene is associated with the risk for hepatocellular carcinoma*. *Carcinogenesis*, 2008. **29**(11): p. 2126-2131.
10. Li, D., et al., *Genetic study of two single nucleotide polymorphisms within corresponding microRNAs and susceptibility to tuberculosis in a Chinese Tibetan and Han population*. *Human Immunology*, 2011. **72**: p. 598-602.
11. Raitoharju, E., et al., *miR-21, miR-210, miR-34a, and miR-146a/b are up-regulated in human atherosclerotic plaques in the Tampere Vascular Study*. *Atherosclerosis*, 2011. **219**: p. 211-217.
12. Xu, J., et al., *Circulating microRNAs: novel biomarkers for cardiovascular diseases*. *Journal of Molecular Medicine*, 2011.
13. Jamaluddin, M.S., et al., *miRNAs: roles and clinical applications in vascular disease*. *Expert Rev Mol Diagn*, 2011. **11**(1): p. 79-89.
14. Zilahi, E., et al., *Increased microRNA-146a/b, TRAF6 gene and decreased IRAK1 gene expressions in the peripheral mononuclear cells of patients with Sjögren's syndrome*. *Immunology Letters*, 2011. **141**: p. 165-168.

15. Jazdzewski, K., et al., *Polymorphic mature microRNAs from passenger strand of pre-miR-146a contribute to thyroid cancer*. Proceedings of the National Academy of Sciences, 2009. **106**(5): p. 1502-1505.
16. Chatzikyriakidou, A., et al., *A polymorphism in the 3'-UTR of interleukin-1 receptor-associated kinase (IRAK1), a target gene of miR-146a, is associated with rheumatoid arthritis susceptibility*. Joint Bone Spine, 2010. **77**(5): p. 411-413.
17. Yue, C., et al., *Polymorphism of the pre-miR-146a is associated with risk of cervical cancer in a Chinese population*. Gynecologic Oncology, 2011. **122**: p. 33-37.
18. Curtale, G., et al., *An emerging player in the adaptive immune response: microRNA-146a is a modulator of IL-2 expression and activation-induced cell death in T lymphocytes*. Blood, 2010. **115**(2): p. 265-273.
19. Taganov, K.D., et al., *NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses*. Proceedings of the National Academy of Sciences, 2006. **103**(33): p. 12481-12486.
20. Mohan, V., J.V. Venkatraman, and R. Pradeepa, *Epidemiology of cardiovascular disease in type 2 diabetes: the Indian scenario*. Journal of Diabetes Science and Technology, 2010. **4**(1): p. 158-170.
21. Murray, C.J. and A.D. Lopez, *Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study*. The Lancet, 1997. **349**: p. 1498-1504.
22. Reddy, K.S. and S. Yusuf, *Emerging Epidemic of Cardiovascular Disease in Developing Countries*. Circulation, 1998. **97**(6): p. 596-601.
23. Seedat, Y.K., *Review: Diabetes mellitus in South African Indians*. The British Journal of Diabetes & Vascular Disease, 2005. **5**(5): p. 249-251.
24. Sharma M, G.M., *Premature coronary artery disease in Indians and its associated risk factors*. Vascular Health and Risk Management, 2005. **1**(3): p. 217-225.
25. Norman R BD, S.M., Pieterse D, Groenwald P., *Revised Burden of Disease Estimates for the comparative risk factor assessment, South Africa 2000*. Cape Town: Medical Research Council 2006., 2006.
26. Sambrook, J. and D.W. Russell, *Rapid isolation of mammalian DNA in Molecular Cloning: A laboratory manual*. 3 ed2001, New York: COLD SPRING HARBOR LABORATORY PRESS.
27. Livak, K.J. and T.D. Schmittgen, *Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta CT$ Method*. Methods, 2001. **25**(4): p. 402-408.
28. Takeda, K. and S. Akira, *TLR signalling pathways*. Seminars in Immunology, 2004. **16**: p. 3-9.
29. Pauley, K.M., et al., *Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients*. Arthritis Research & Therapy, 2008. **10**(4).

30. Hirschfield, G.M. and M.B. Pepys, *C-reactive protein and cardiovascular disease: new insights from an old molecule*. QJM, 2003. **96**(11): p. 793-807.
31. Shen, J., et al., *A functional polymorphism in the miR-146a gene and age of familial breast/ovarian cancer diagnosis*. Carcinogenesis, 2008. **29**(10): p. 1963-1966.

CHAPTER 7

CONCLUSION

Indian populations throughout the world show an unusually high prevalence of early-onset CAD compared to other ethnicities (Rajeshwari, Nicklas et al. 2005; Sharma M 2005; Aggarwal, Aggarwal et al. 2012; Khan 2013). South African Indians have a much higher mortality rate due to CAD compared to Black South Africans (Norman, Schneider et al. 2006). Until recently, genetic and epigenetic studies in CAD in developing countries such as SA have resulted in minimal literature being available. This study is the first to investigate genetic and miRNA polymorphisms in SA Indians with CAD. We assessed SNPs in four candidate genes; MTHFR, SIRT1, miR-146a, and miR-499; all of which have been implicated in chronic inflammatory diseases such as CAD, but have never been investigated in SA Indians.

The MTHFR gene encodes for a protein with important enzymatic functions in folate and methyl-group metabolism (Botezatu, Socolov et al. 2013). The enzyme irreversibly catalyses the reduction of 5',10'-methylenetetrahydrofolate to 5'-methyltetrahydrofolate; a major form of circulating folate, and the precursor of SAM - the universal methyl donor for several biological methylation reactions (Kluijtmans and Whitehead 2001; Friso, Girelli et al. 2002). The common MTHFR C677T SNP has been widely investigated and is known to affect enzyme activity (Vasisht, Gulati et al. 2002; Oyama, Kawakami et al. 2004; Sastry, Golla et al. 2011; Wang, Pei et al. 2012).

An important finding in this study was the significantly higher prevalence of the MTHFR 677 variant allele in CAD patients compared with Indian controls. In addition, it was also observed that Indian controls presented with a higher frequency of the variant allele when compared to Black controls. This is of significance since the variant allele has been associated with increased levels of Hcy, an independent risk factor for CAD (Mehlig, Leander et al. 2013). The higher frequency of the MTHFR 677 variant allele in SA Indians may be a contributing factor to the higher risk profile for the development of early-onset CAD in this population. In Black South Africans, the low prevalence of the MTHFR 677 variant allele may confer protection against CAD.

Further, the results of this study support the findings of a recent study that investigated the frequency of the MTHFR C677T SNP and its effect on homocysteine levels in another cohort of Black South Africans. It was observed that 15.2%, 0.8%, and 84% of the study population harboured the MTHFR 677 CT, TT, and CC genotypes respectively (Nienaber-Rousseau, Ellis et al. 2013). Subjects with the MTHFR 677 homozygous variant genotype had the highest Hcy levels while carriers of the CC genotype had the lowest Hcy levels. The low incidence of the MTHFR 677 TT genotype amongst Black South Africans was speculated to play a protective role in this population against risk of Hcy-associated disease (Nienaber-Rousseau, Ellis et al. 2013). The results of this study and that of

Nienaber-Rousseau *et al.* 2013 show a consistency with regard to the low prevalence of the MTHFR TT genotype in Black South Africans.

Like MTHFR, SIRT1 has also been identified as a candidate that affects the epigenetic mechanisms of CAD (Cui, Wang *et al.* 2012). Sirtuin 1 is a class III histone deacetylase that regulates several important metabolic and physiological pathways. In addition to targeting histones, numerous non-histone SIRT1 target proteins such as adenosine monophosphate activated protein kinase (AMPK), FoxO, NF- κ B, eNOS, p53, peroxisome proliferator-activated receptor-gamma (PPAR- γ) and its coactivator PGC-1 α , have been identified (Tomé-Carneiro, Larrosa *et al.* 2013; Ma and Li 2015). In the cardiovascular system, SIRT1 has been recognised as a key regulator of vascular endothelial homeostasis, and also regulates angiogenesis, endothelial senescence, and dysfunction. It serves a critical role in RCT and reduces the risk of CVD (Ma and Li 2015).

This study compared the role of two SIRT1 SNPs, rs1467568 and rs789583, in SA Indians with CAD to Indian and Black controls. No difference was observed in the distribution of SIRT1 SNPs between CAD patients and control groups. The variant allele was more frequent in Indian controls compared to Black controls, but this did not reach statistical significance. Previous studies have shown that SIRT1 SNPs are associated with BMI and obesity, blood pressure, dyslipidaemia, inflammation, and coronary artery calcification (Peeters, Beckers *et al.* 2008; Zillikens, van Meurs *et al.* 2009; Shimoyama, Suzuki *et al.* 2011; Botden, Zillikens *et al.* 2012; Shimoyama, Mitsuda *et al.* 2012). This study observed no association between the SIRT1 SNPs and BMI, total cholesterol, LDL, HDL, triglycerides, fasting glucose, fasting insulin, HbA1c, hsCRP, or IL-6 in CAD patients and control groups.

The frequency of rs7895833 and rs1467568 vary among different ethnicities, and may be a reason for differing disease patterns among populations. The frequency of the rs7895833 A allele was relatively low (0.29) in Japanese compared to Dutch, Turkish and Caucasians who had similar allele frequencies (0.80, 0.85, and 0.80 respectively) (Zillikens, van Meurs *et al.* 2009; Maeda, Koya *et al.* 2011; Shimoyama, Suzuki *et al.* 2011) The A allele of rs1467568 showed marked difference in frequency between the European (0.25) and Japanese (0.84) (Maeda, Koya *et al.* 2011). To date, there have been no published studies investigating rs7895833 and rs1467568 in Indians with CAD to validate the findings of the present study.

It's important to note that metabolic effects of SIRT1 reported in literature have mainly been observed under conditions of calorie restriction (CR) (Cohen 2004; Chen, Bruno *et al.* 2008; Naqvi, Hoffman *et al.* 2010; Botden, Zillikens *et al.* 2012), while the present study population was not subjected to CR. It is possible that associations between SIRT1 variants and CAD risk can only be found when interactions with environmental factors such as CR are taken into account (Peeters, Beckers *et al.* 2008).

A more recent advance in epigenetic research has been the study of miRNAs. These small noncoding RNA molecules negatively regulate target gene expression by inhibiting mRNA translation or promoting mRNA degradation (Jamaluddin, Weakly et al. 2011). MicroRNA polymorphisms were shown to affect miRNA expression and/or miRNA-mRNA interaction, and have been associated with human diseases (Chen, Song et al. 2008; Ardekani and Naeini 2010; Li, Chen et al. 2010). Since CAD is a chronic inflammatory disease, SNPs in inflammatory associated miRNAs were of interest in this study. Two such SNPs, rs3746444 and rs2010164, in miR-499 and miR-146a respectively were assessed.

MicroRNA-499 plays a critical role in inflammation by targeting several cytokines that play a role in the pathogenesis of CAD. These targets include IL-2, IL-2 β , IL-6, IL-17, IL-17R β , IL-18, IL-21, and IL-23a (Yang, Chen et al. 2012). The variant (G) allele of a common miR-499 SNP, rs3746444, results in a less stable secondary structure than the ancestral (A) allele and affects the binding to target mRNAs (Akkız, Bayram et al. 2011).

The miR-499 variant allele was more frequent in CAD patients compared to Indian controls, almost reaching statistical significance. The total Indian group presented with a higher frequency of the rs3746444 G allele compared to the total Black population but this was not statistically significant. Since miR-499 targets inflammatory cytokines, the levels of IL-6 and hsCRP were then assessed. A trend was observed in CAD patients harboring the variant genotypes having elevated IL-6 and hsCRP compared to those with the wild type genotypes. The change from A-to-G in the seed sequence of miR-499 may interfere with its binding to and interaction with targets such as IL-6. CRP is under the direct influence of IL-6, which may explain why we observed increased levels of both (IL-6 and hsCRP) in patients with the variant genotype.

A comprehensive literature search revealed only two published studies regarding miR-499 and CAD. Of these two studies, Zhi *et al* found that the GG genotype was associated with a 3.23-fold increased risk of CAD in a Chinese Han population (Zhi, Wang et al. 2012). In contrast, Xiong *et al* found no association between rs3746444 and CAD in another Chinese Han population (Xiong, Cho et al. 2014). Our findings may implicate miR-499 as a target in the regulation of inflammation in CAD, but a larger follow up study investigating the functional significance of this miR-499 SNP may provide additional clarification.

A major finding in this study was the role of a common miR-146a SNP, rs rs2910164, in CAD patients. The frequency of genotypes was similar between CAD patients and Indian controls. Interestingly, a significant 6.25-fold increase in miR-146a levels was observed in CAD patients with the CC genotype relative to controls and patients with the wildtype variant. We then investigated the targets of miR-146a, IRAK-1 and TRAF-6, and found that the expression of these targets was significantly decreased in CAD patients with the CC genotype. Both miR-146a targets act

downstream of the NF- κ B-dependant TIR signalling pathway (Chatzikyriakidou, Voulgari et al. 2010). The lowest levels of NF- κ B were found in patients with the homozygous C allele compared to the heterozygous GC and wildtype variants. In addition, these patients (CC genotype) also had the lowest hsCRP compared to those with the other genotypes.

Since high levels of pro-inflammatory cytokines contribute to the chronic inflammation seen in CAD, the CC genotype serves a protective function by increasing miR-146a levels and reducing inflammation. The results of this study implicate a role for miR-146a in TIR signalling via a negative feedback mechanism involving the attenuation of NF- κ B by downregulation of IRAK-1 and TRAF-6. These findings suggest miR-146a as a target for therapy towards lowering inflammation in CAD patients.

There have been substantial advances recently in elucidating the genetic background of many complex diseases, including CAD but much, if not most, is yet to be discovered – especially in the Indian population. Identifying individuals at risk for the development of CAD is integral for primary prevention of the disease. Genetics in combination with clinical parameters may help us to understand pathogenesis of disease and possibly will improve the prediction of CAD.

Although recruitment numbers were adequate to detect statistical differences, follow-up studies with a larger sample size may provide further clarification. Nevertheless, the findings presented provide improved insight into how underlying genetic and epigenetic factors influence CAD in SA Indians. Hopefully, such insight may be translated into future interventions capable of providing CAD patients in general, a longer and healthier life.

Taken together, the findings of this study provide insight into four candidate genes for CAD in young South African Indians and Blacks. This was the first investigation of genetic (MTHFR and SIRT1) and epigenetic (miR-146a and miR-499) polymorphisms in young SA Indians with CAD. The frequency of variant alleles was predominant in SA Indians and may predispose towards the development of early-onset CAD by regulating the pathogenesis of CAD.

References

- Aggarwal, A., S. Aggarwal, et al. (2012). "A retrospective case-control study of modifiable risk factors and cutaneous markers in Indian patients with young coronary artery disease." Journal of the Royal Society of Medicine Cardiovascular Disease **1**(8): 39-45.
- Akkız, H., S. Bayram, et al. (2011). "Genetic Variation in the MicroRNA-499 Gene and Hepatocellular Carcinoma Risk in a Turkish Population: Lack of Any Association in a Case-Control Study." Asian Pacific Journal of Cancer Prevention **12**: 3107-3112.
- Ardekani, A. M. and M. M. Naeini (2010). "The Role of MicroRNAs in Human Diseases." Avicenna Journal of Medical Biotechnology **2**(4): 161-179.
- Botden, I. P. G., M. C. Zillikens, et al. (2012). "Variants in the SIRT1 Gene May Affect Diabetes Risk in Interaction With Prenatal Exposure to Famine." Diabetes Care **35**(2): 424-426.
- Botezatu, A., D. Socolov, et al. (2013). "Methylenetetrahydrofolate reductase (MTHFR) polymorphisms and promoter methylation in cervical oncogenic lesions and cancer." Journal of Cellular and Molecular Medicine **17**(4): 543-549.
- Chatzikyriakidou, A., P. V. Voulgari, et al. (2010). "A polymorphism in the 3'-UTR of interleukin-1 receptor-associated kinase (IRAK1), a target gene of miR-146a, is associated with rheumatoid arthritis susceptibility." Joint Bone Spine **77**(5): 411-413.
- Chen, D., J. Bruno, et al. (2008). "Tissue-specific regulation of SIRT1 by calorie restriction." Genes & Development **22**(13): 1753-1757.
- Chen, K., F. Song, et al. (2008). "Polymorphisms in microRNA targets: a gold mine for molecular epidemiology." Carcinogenesis **29**(7): 1306-1311.
- Cohen, H. Y. (2004). "Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase." Science **305**: 390-392.
- Cui, Y., H. Wang, et al. (2012). "Genetic analysis of the SIRT1 gene promoter in myocardial infarction." Biochemical and Biophysical Research Communications **426**(2): 232-236.
- Friso, S., D. Girelli, et al. (2002). "A1298C methylenetetrahydrofolate reductase mutation and coronary artery disease: relationships with C677T polymorphism and homocysteine/folate metabolism." Clinical and Experimental Medicine **2**(1): 7-12.
- Jamaluddin, M. S., S. M. Weakly, et al. (2011). "miRNAs: roles and clinical applications in vascular disease." Expert Rev Mol Diagn **11**(1): 79-89.
- Khan, S. (2013). Telomere Dynamics in Atherosclerosis. Doctor of Philosophy, University of KwaZulu-Natal.
- Kluijtmans, L. A. J. and A. S. Whitehead (2001). "Methylenetetrahydrofolate reductase genotypes and predisposition to atherothrombotic disease." European Heart Journal **22**: 294-299.
- Li, L., X. Chen, et al. (2010). "MicroRNA-146a and human disease." Scandinavian Journal of Immunology **71**: 227-231.

- Ma, L. and Y. Li (2015). "SIRT1: Role in cardiovascular biology." Clinica Chimica Acta **440**(0): 8-15.
- Maeda, S., D. Koya, et al. (2011). "Association between single nucleotide polymorphisms within genes encoding sirtuin families and diabetic nephropathy in Japanese subjects with type 2 diabetes." Clinical and Experimental Nephrology **15**(3): 381-390.
- Mehlig, K., K. Leander, et al. (2013). "The association between plasma homocysteine and coronary heart disease is modified by the MTHFR 677C>T polymorphism." Heart (0): 1-5.
- Naqvi, A., T. A. Hoffman, et al. (2010). "A single-nucleotide variation in a p53-binding site affects nutrient-sensitive human SIRT1 expression." Human Molecular Genetics **19**(21): 4123-4133.
- Nienaber-Rousseau, C., S. M. Ellis, et al. (2013). "Gene–environment and gene–gene interactions of specific MTHFR, MTR and CBS gene variants in relation to homocysteine in black South Africans." Gene **530**(1): 113-118.
- Norman, R. B. D., M. Schneider, et al. (2006). Revised Burden of Disease Estimates for the comparative risk factor assessment, South Africa 2000. Cape Town: Medical Research Council 2006.
- Oyama, K., K. Kawakami, et al. (2004). "The Association Between Methylenetetrahydrofolate Reductase Polymorphism and Promoter Methylation in Proximal Colon Cancer." Anticancer Research **24**(2B): 649-654.
- Peeters, A., S. Beckers, et al. (2008). "Association of SIRT1 gene variation with visceral obesity." Human Genetics **124**(4): 431-436.
- Rajeshwari, R., T. A. Nicklas, et al. (2005). "Cardiovascular diseases—a major health risk in Asian Indians." Nutrition research (New York, N.Y.) **25**(6): 515-533.
- Sastry, B. K. S., J. Golla, et al. (2011). "Genetic interactions between MTHFR (C677T), methionine synthase (A2756G, C2758G) variants with vitamin B12 and folic acid determine susceptibility to premature coronary artery disease in Indian population." Journal of Cardiovascular Disease Research **2**(3): 156-163.
- Sharma M, G. M. (2005). "Premature coronary artery disease in Indians and its associated risk factors." Vascular Health and Risk Management **1**(3): 217-225.
- Shimoyama, Y., Y. Mitsuda, et al. (2012). "SIRTUIN 1 Gene Polymorphisms are Associated With Cholesterol Metabolism and Coronary Artery Calcification in Japanese Hemodialysis Patients." Journal of Renal nutrition **22**(1): 114 - 119.
- Shimoyama, Y., K. Suzuki, et al. (2011). "Sirtuin 1 gene polymorphisms are associated with body fat and blood pressure in Japanese." Translational Research **157**(6): 339-347.
- Tomé-Carneiro, J., M. Larrosa, et al. (2013). "One-year supplementation with a grape extract containing resveratrol modulates inflammatory-related microRNAs and cytokines expression in peripheral blood mononuclear cells of type 2 diabetes and hypertensive patients with coronary artery disease." Pharmacological Research **72**: 69-82.

- Vasisht, S., R. Gulati, et al. (2002). "Polymorphism (C677T) in the 5,10-Methylenetetrahydrofolate reductase (MTHFR) Gene: A Preliminary Study in North Indian Men." Indian Journal of Clinical Biochemistry **17**(1): 99-107.
- Wang, Y., L. Pei, et al. (2012). "Is the prevalence of MTHFR C677T polymorphism associated with ultraviolet radiation in Eurasia?" Journal of Human Genetics **57**(12): 780-786.
- Xiong, X. D., M. Cho, et al. (2014). "A common variant in pre-miR-146 is associated with coronary artery disease risk and its mature miRNA expression." Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis(1386-1964 (Print)).
- Yang, B., J. Chen, et al. (2012). "Association of polymorphisms in pre-miRNA with inflammatory biomarkers in rheumatoid arthritis in the Chinese Han population." Human Immunology **73**(1): 101-106.
- Zhi, H., L. Wang, et al. (2012). "Polymorphisms of miRNAs genes are associated with the risk and prognosis of coronary artery disease." Clinical Research in Cardiology **101**: 289-296.
- Zillikens, M. C., J. B. J. van Meurs, et al. (2009). "SIRT1 Genetic Variation Is Related to BMI and Risk of Obesity." Diabetes **58**(12): 2828-2834.

APPENDIX 1

Ethics Statement



**UNIVERSITY OF
KWAZULU-NATAL**
INYUVESI
YAKWAZULU-NATALI

23 December 2014

Ms Prithiksha Ramkaran
P O Box 3333
Stanger
4450
Prithiksharamkaran@gmail.com

PROTOCOL: An epigenetic evaluation using microRNA and histone modifications in young South African Coronary Artery Disease patients. REF: BED67/14

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 07 February 2014.

The study was provisionally approved pending appropriate responses to queries raised. Your responses received on 22 December 2014 to queries raised on 19 June 2014 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 23 December 2014.

This approval is valid for one year from 23 December 2014. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2004), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at <http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx>.

BREC is registered with the South African National Health Research Ethics Council (REC 290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its meeting taking place on 10 February 2015.

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

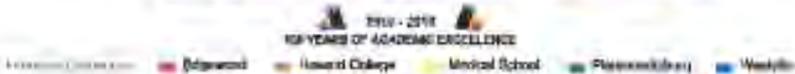


Professor D.R. Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Professor D.R. Wassenaar (Chair)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag 354001 | Durban 4001

Telephone: +27 (0) 31 260 5490 Fax: +27 (0) 31 260 6029 Email: brec@ukzn.ac.za
Website: <http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx>

2014 - 2018
10 YEARS OF ACADEMIC EXCELLENCE



APPENDIX 2

Polymerase Chain Reaction

The principle of polymerase chain reaction (PCR) is based on the exponential amplification of a particular DNA sequence using specially designed primers, deoxynucleoside triphosphates (dNTP), DNA polymerase (usually derived from *Thermus aquaticus* (*Taq*)), buffer solution, magnesium chloride ($MgCl_2$), and a DNA template (Figure 1). The process relies on thermal cycling, consisting of repeated heating cooling cycles, and involves three major steps i.e. denaturation, annealing, and extension.

Denaturation occurs at approximately $95^{\circ}C$. Double-stranded DNA (dsDNA) is melted, forming single-stranded DNA templates. Temperature is lowered (between $55-60^{\circ}C$) and the primers are allowed to anneal to the 3' ends of each single-stranded DNA template. Extension of DNA occurs at $72^{\circ}C$, the optimum temperature at which *Taq* polymerase functions to extend the target DNA sequence by the addition of dNTPs from the 3' end of the annealed primer.

The steps are repeated for 20 to 40 cycles, and at the end of each cycle DNA is amplified two-fold. The PCR product is run on an agarose gel by means of electrophoresis. Visualization is enabled by staining with ethidium bromide and UV light exposure. A DNA ladder (also known as a molecular weight marker) may be run alongside to determine the size of the PCR product.

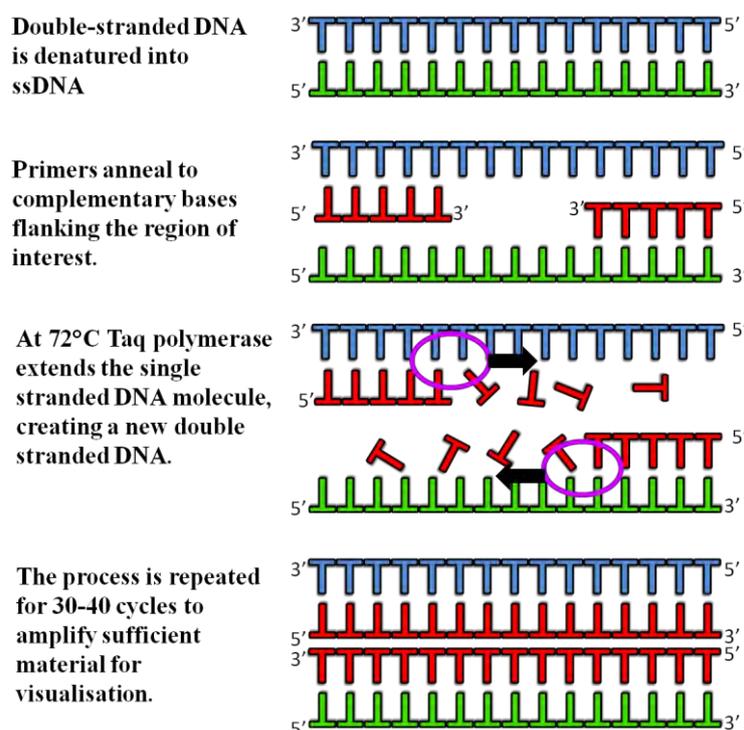


Figure 1 The Polymerase Chain Reaction (Author's own work)

Optimisation of Polymerase Chain Reaction

Major components of the PCR (primers, MgCl₂ and GoTaq® DNA polymerase) were optimised to ensure an efficient and specific amplification process. Primer sets were optimised first by varying the concentrations between 10 and 100 µmol, while all other components were kept at a standard concentration. Once the PCR was completed, amplicons were verified by electrophoresis (150 V; 50 min) on agarose gel (1.8%, 0.5 mg/ml ethidium bromide) and visualized by ultra violet (UV) light and digitally photographed using a gel documentation system (Uvitech Alliance 2.7).

The reaction that had the least non-specific binding, and produced a clear band of the target DNA was chosen. This concentration was used in the optimisation of MgCl₂. Finally *Taq* polymerase was optimised using the optimal primer and MgCl₂ concentrations. MgCl₂ and *Taq* polymerase concentrations ranged from 0.5 to 3 mM and 0.1 to 0.5 units (U) respectively.

Since no single protocol is appropriate to all situations, each new PCR application required optimisation. Optimised concentrations for each PCR component for each SNP investigated can be found in below (Table 1 – Table 3):

Table 1 miR-146a G/C SNP (rs2010164)

Optimised Conditions (Reaction volume = 30µl)	Reaction	Optimised concentrations
DNA template		10 ng/µl
MgCl ₂		2.5 mmol
<i>Taq</i> polymerase		0.2U
dNTP		200 µM of each
Forward primer (100 µmol)		40 µmol
Reverse primer (100 µmol)		40 µmol
5× Green GoTaq® Flexi Buffer		1×
Nuclease-free H ₂ O		
Optimised Cycling Conditions (30 cycles)		
Denaturation		94°C for 10 minutes
		94°C for 30 seconds
Annealing		65°C for 30 seconds
Final extension		72°C for 7 minutes

Table 2 MTHFR C/T SNP (rs1801133)

Optimised Conditions (Reaction volume = 25µl)	Reaction	Optimised concentrations
DNA template		10 ng/µl
MgCl ₂		2.5 mmol
<i>Taq</i> polymerase		0.2U
dNTP		200 µM of each
Forward primer (100 pmol)		20 pmol
Reverse primer (100 pmol)		20 pmol
5× Green GoTaq® Flexi Buffer		1×
Nuclease-free H ₂ O		
Optimised Cycling Conditions (35 cycles)		
Denaturation		95°C for 5 minutes
		95°C for 1 minute
Annealing		59°C for 1 minute
Extension		72°C for 2 minutes
Final extension		72°C for 7 minutes

Table 3 miR-499 G/C SNP (rs3746444)

Optimised Conditions (Reaction volume = 25µl)	Reaction	Optimised concentrations
DNA template		10 ng/µl
MgCl ₂		2.5 mmol
<i>Taq</i> polymerase		0.5Unit
dNTP		200 µM of each
Forward primer (100 µmol)		40 µmol
Reverse primer (100 µmol)		40 µmol
5× Green GoTaq® Flexi Buffer		1×
Nuclease-free H ₂ O		
Optimised Cycling Conditions (34 cycles)		
Denaturation		94°C for 5 minutes
		94°C for 1 minute
Annealing		59°C for 30 seconds
Extension		72°C for 2 minutes
Final extension		72°C for 7 minutes

APPENDIX 3

Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP) is a method that exploits variations in homologous DNA sequences. Restriction enzymes recognize specific nucleotide sequences on a DNA strand and cleave at precise points. Following PCR, the PCR products are digested by a specific restriction enzyme to analyse the presence of a polymorphism. The consensus sequences and cleavage sites for each enzyme is unique, shown below (Table 4 – Table 6):

Table 4 Consensus sequence and cleavage site (arrows) for *Sac* I:

Restriction Enzyme	Consensus Sequence
<i>Sac</i> I	5'...G A G C T ↓ C... 3' 3'...C ↑ T C G A G... 5'

Table 5 Consensus sequence and cleavage site (arrows) for *Hinf* I:

Restriction Enzyme	Consensus Sequence
<i>Hinf</i> I	5'...G ↓ A N T C... 3' 3'...C T N A ↑ G... 5'

Table 6 Consensus sequence and cleavage site (arrows) for *Bcl* I:

Restriction Enzyme	Consensus Sequence
<i>Bcl</i> I	5'...T ↓ G A T C A... 3' 3'...A C T A G ↑ T... 5'

Overnight digestion was performed (at 37°C for *Sac* I and *Hinf* I; and at 55°C for *Bcl* I) in 25µL (15µL PCR product, 4.5µL enzyme-specific Buffer and 0.5µL (5U) restriction enzyme). Restriction fragments were electrophoresed on an agarose gel (3%, 0.5mg/ml ethidium bromide) and visualized (Figure 2).

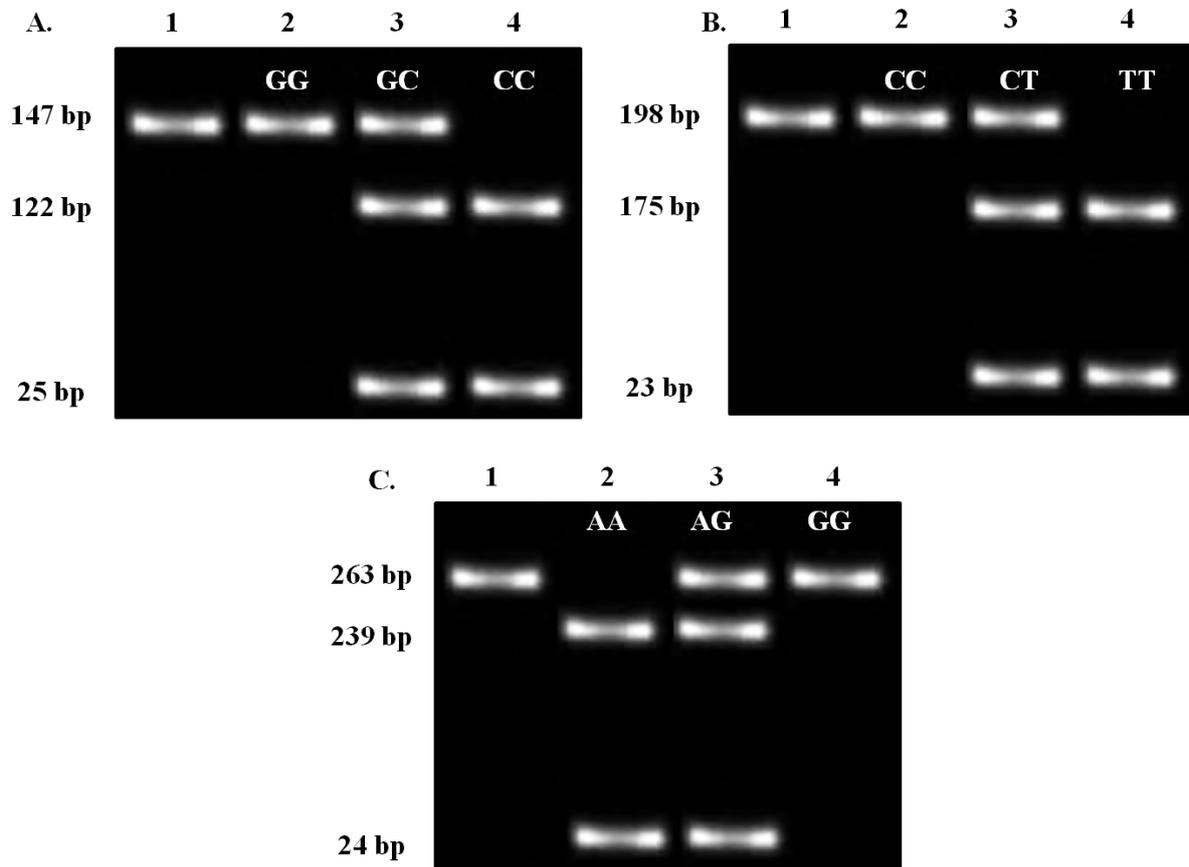


Figure 2 Diagrammatic representations of PCR-RFLP product sizes and corresponding genotypes for A. miR-146a rs2910164, B. MTHFR rs1801133, and C. miR-499 rs3746444. Lane 1 represents the PCR product

APPENDIX 4

Synthesis of cDNA

Total RNA was used to prepare copy DNA (cDNA) using the miScript II RT kit (miScript Reverse Transcriptase Mix, 10× miScript Nucleics Mix, 5× miScript HiSpec Buffer; Qiagen, Catalog no. 218161) following the manufacturer’s instructions (Figure 3). Components were added according to Table below (Table 7).

Table 7 Components of reverse transcription reaction

Component	Volume/reaction
5× miScript HiSpec Buffer	4µl
10× miScript Nucleics Mix	2µl
RNase-free water	10µl
miScript Reverse Transcriptase Mix	2µl
Template RNA	2µl
Total volume	20µl

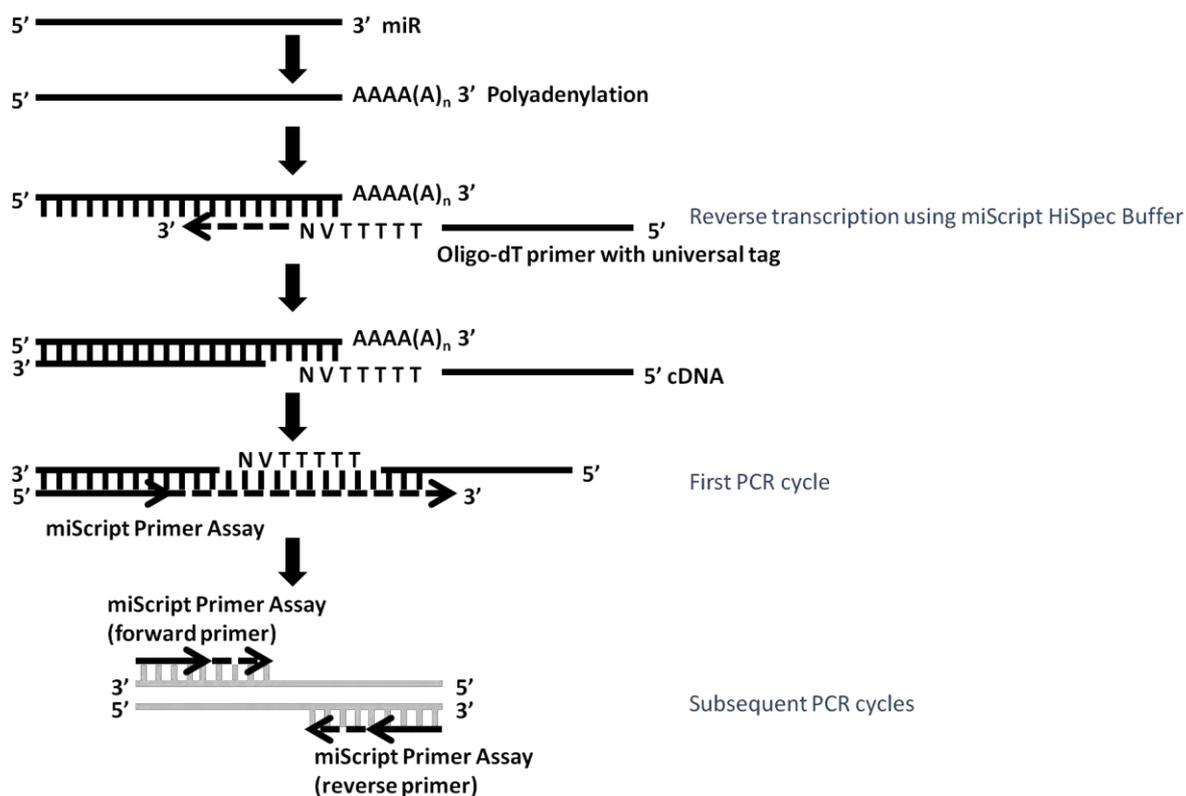


Figure 3 Synthesis of cDNA using the miScript II RT kit (Qiagen)

APPENDIX 5

Quantitative Polymerase Chain Reaction

Quantitative PCR (qPCR) is a technique based on the general principle of PCR and is used to simultaneously amplify and quantify the target DNA. Amplified DNA is detected as the reaction progresses in *real-time*, and hence qPCR is also known as real-time PCR. This method does not require the end-detection of the product as in the case of traditional PCR where the product is run on a gel via electrophoresis. Quantitative PCR incorporates SYBR Green, a dye used as a nucleic acid stain that binds to dsDNA, which emits fluorescent intensity proportional to the amount of dsDNA.

Quantitative PCR was used to determine the levels of miR-146a for each genotype in CAD patients and controls. A reaction mix for detection of mature miR-146a was prepared and subjected to the cycling conditions as follows (Table 8 and Table 9):

Table 8 Reaction mix for detection of mature miR-146a

Component	Volume/reaction
2× QuantiTect SYBR Green PCR Master Mix	12.5 µl
10× miScript Universal Primer Assay	2.5 µl
10× miScript Primer Assay	2.5 µl
RNase-free water	5.5 µl
Template cDNA	2 µl
Total volume	25 µl

Table 9 Cycling conditions for the quantitative analysis of mature miR-146a

Step	Time	Temperature
Initial activation step	15 min	95°C
3-step cycling:		
Denaturation	15 sec	94°C
Annealing	30 sec	55°C
Extension	30 sec	70°C
Cycle number	40 cycles	

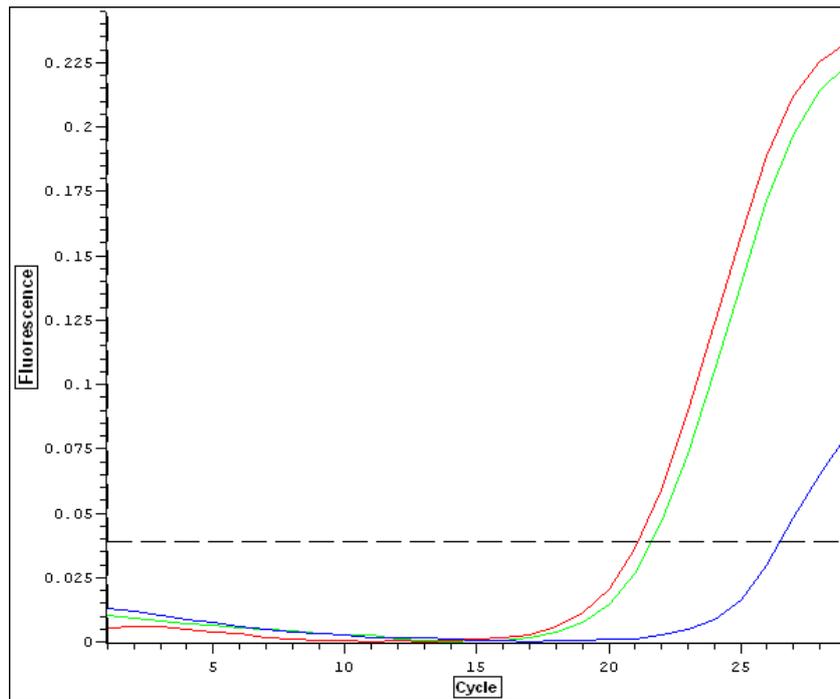


Figure 4 miR-146a levels in each miR-146a rs2910164 genotype in CAD patients. The CC genotype had the highest miR-146a expression, and therefore the lowest CT value compared to the other genotypes (red: GG, green: GC, blue: CC)

Analysis of Quantitative Polymerase Chain Reaction Results

The comparative threshold cycle (C_T) method was used to assess the relative changes in miR-146a expression. $2^{-\Delta\Delta C_T}$ represents the fold change in miR-146a expression between sample groups (Figure 4). A negative control without a genomic DNA template was run with the positive samples to assess the overall specificity of the reaction (Livak and Schmittgen 2001). The figure below is a table extracted directly from the original article, and highlights an example of relative quantification (Figure 5).

TABLE 1
Treatment of Replicate Data Where Target and Reference Are Amplified in Separate Wells^a

Tissue	<i>c-myc</i> C _T	GAPDH C _T	ΔC_T (Avg. <i>c-myc</i> C _T - Avg. GAPDH C _T)	$\Delta\Delta C_T$ (Avg. ΔC_T - Avg. $\Delta C_{T,Brain}$)	Normalized <i>c-myc</i> amount relative to brain $2^{-\Delta\Delta C_T}$
Brain	30.72	23.70			
	30.34	23.56			
	30.58	23.47			
	30.34	23.65			
	30.50	23.69			
	30.43	23.68			
Average	30.49 ± 0.15	23.63 ± 0.09	6.86 ± 0.17	0.00 ± 0.17	1.0 (0.9–1.1)
Kidney	27.06	22.76			
	27.03	22.61			
	27.03	22.62			
	27.10	22.60			
	26.99	22.61			
	26.94	22.76			
Average	27.03 ± 0.06	22.66 ± 0.08	4.37 ± 0.10	-2.50 ± 0.10	5.6 (5.3–6.0)

^a Total RNA from human brain and kidney were purchased from Clontech. Using reverse transcriptase, cDNA was synthesized from 1 μ g total RNA. Aliquots of cDNA were used as template for real-time PCR reactions containing either primers and probe for *c-myc* or primers and probe for GAPDH. Each reaction contained cDNA derived from 10 ng total RNA. Six replicates of each reaction were performed.

Figure 5 An example of relative quantification using the comparative threshold cycle (C_T) method (Livak and Schmittgen 2001).

APPENDIX 6

Protein Isolation

Cytobuster™ reagent (Novagen) supplemented with protease and phosphatase inhibitors (Roche, 05892791001 and 04906837001 respectively) was used for protein isolation. Cytobuster (200µl) was added to PBMCs (4°C, 10 min) and centrifuged (180 x g; 4°C, 10 min) to obtain a crude protein extract. Protein samples were quantified using the bicinchoninic acid (BCA) assay.

The Bicinchoninic Acid Assay

The bicinchoninic acid assay (BCA) is based on the reduction of Cu^{2+} to Cu^+ by protein in an alkaline medium (the biuret reaction). The Cu^+ is then detected by reaction with BCA, and results in the development of a purple-colored product with maximum absorbance at 562nm.

Serial dilutions of Bovine serum albumin (BSA) were prepared (0mg/ml, 0.2mg/ml, 0.4mg/ml, 0.6mg/ml, 0.8mg/ml, and 1mg/ml) to serve as standard concentrations (Figure 6). 25µl of each sample and the relevant standards were added to appropriately labelled wells, followed by the addition of 200µl BCA working solution (4µl CuSO_4 and 198µl BCA) into each well. The plate was incubated at 37°C for 30 minutes. Absorbance was read at 562nm using a spectrophotometer. Concentration of each protein sample was calculated from a standard curve constructed using the absorbencies of the standards. Proteins were standardised to 0.5mg/ml.

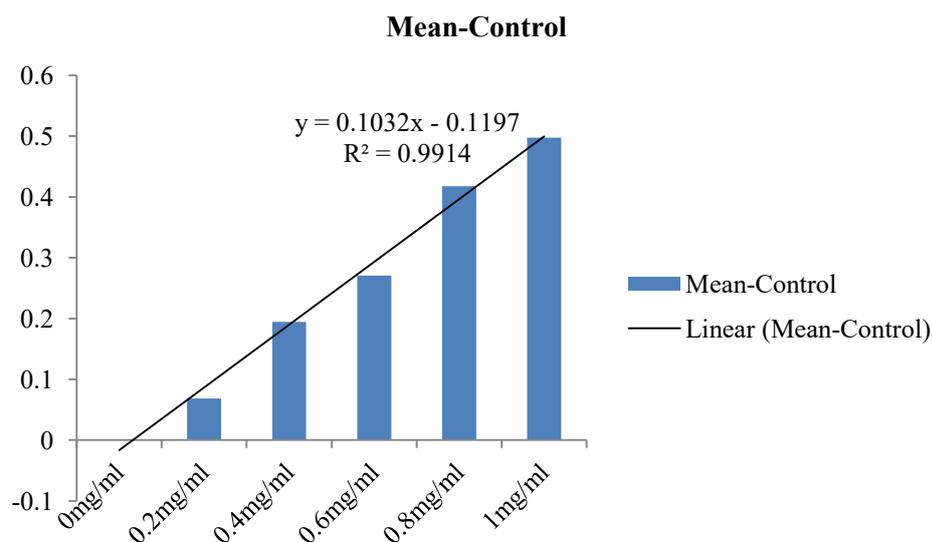


Figure 6 Protein standardisation by the BCA

Preparation of samples for Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Laemlii sample buffer (0.5M Tris (pH 6.8), glycerol, 10%SDS, 0.5% bromophenol blue, β -mercaptoethanol, dH₂O) was prepared and added to protein samples (1:1). Samples were boiled at 95°C for 5 minutes to activate the β -mercaptoethanol and SDS to allow for reduction and unfolding of proteins.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis and Western Blot

Western blotting is a technique used to detect and analyse proteins. Proteins are separated on two types of agarose gel (stacking and resolving) via electrophoresis based on their molecular weight. The results are then transferred onto a membrane and blocked with 1% BSA to prevent nonspecific binding of the antibodies. The membrane is incubated with primary and corresponding secondary antibodies (usually labelled with horseradish peroxidase) which are specific to the protein of interest. The unbound antibody is washed off, leaving only the bound antibody. Detection of the protein by chemiluminescence is enabled by the oxidation of luminol (in the detection reagent) in the presence of peroxide, resulting in light emission.

Samples (25 μ l) were electrophoresed on 7.5% SDS-PAGE gels and thereafter transferred to nitrocellulose membranes using the Trans-Blot[®] Turbo[™] Transfer system (BIORAD). Membranes

were blocked with 5% BSA in Tris buffer saline (20mM Tris-HCl (pH 7.4), 500mM NaCl and 0.01% Tween 20 (TBST)) for 1 hour, and incubated with primary antibody (IRAK-1; Cell Signalling Technology 4769, TRAF-6; Cell Signalling Technology 8347, NF- κ B; ab17742, and β -actin; ab8226, 1:1000) in 1% BSA in TBST at 4°C overnight. Membranes were then washed (TBST, 4× 15 min) and treated with secondary antibody (Anti-rabbit IgG, HRP-linked; Cell Signalling Technology 4769, 1:2000) for 1 hour at room temperature. Membranes were washed again (TBST, 4× 15min) and protein expression was detected by the LumiGLO[®] chemiluminescent substrate system (KPL) with the Alliance 2.7 Image Documentation System (UViTech).

Analysis of Western Blot

Protein bands were standardised against β -actin, and analyzed with the UViBand Advanced Image Analysis software (UViTech v12.14). The results were expressed as mean relative band intensity (RBI).

APPENDIX 7

Interleukin-6 Enzyme-linked immunosorbent assay

Human IL-6 enzyme-linked immunosorbent assay (ELISA) Kit II (BD OptEIA™) was used to measure the IL-6 serum levels of patients and controls. As per user manual, ELISA diluent (12ml buffered protein base, 0.09% sodium azide) was added into a 96 well microtitre plate coated with anti-human IL-6 monoclonal antibody. A serial two fold dilution ranging from 0pg/ml to 250pg/ml was prepared (Table 10). Standards and samples were added into appropriate wells. Following incubation (2h, RT) wells were rinsed with wash buffer (1× detergent solution with ProClin™-150). Working detector (containing Streptavidin-horseradish peroxidase and Biotinylated anti-human IL-6 monoclonal antibody) was added into each well and incubated (1h, RT, dark). Wells were rinsed with wash buffer, seven times, followed by the addition of TMB One-Step Substrate Reagent (3,3',5,5'-tetramethylbenzidine) and incubated (30min, RT, dark). Following incubation, stop solution (1M phosphoric acid) was added, and absorbance measured at 450nm (reference: 570nm) with a microplate reader (Bio-Tek μQuant, United States). Serum concentrations of IL-6 were calculated by extrapolation from the standard curve (Figure 7).

Table 10 Preparation of samples of known concentration for the IL-6 ELISA

Standard concentration	Volume IL-6	Volume Distilled Water
150 pmol	50.0 μl	50.00 μl
75 pmol	25.0 μl	45.00 μl
37.5 pmol	12.5 μl	87.50 μl
18.8 pmol	6.52 μl	93.75 μl
9.4 pmol	3.13 μl	96.87 μl
4.7 pmol	1.56 μl	98.44 μl
0.00 pmol	0.00 μl	100.00 μl

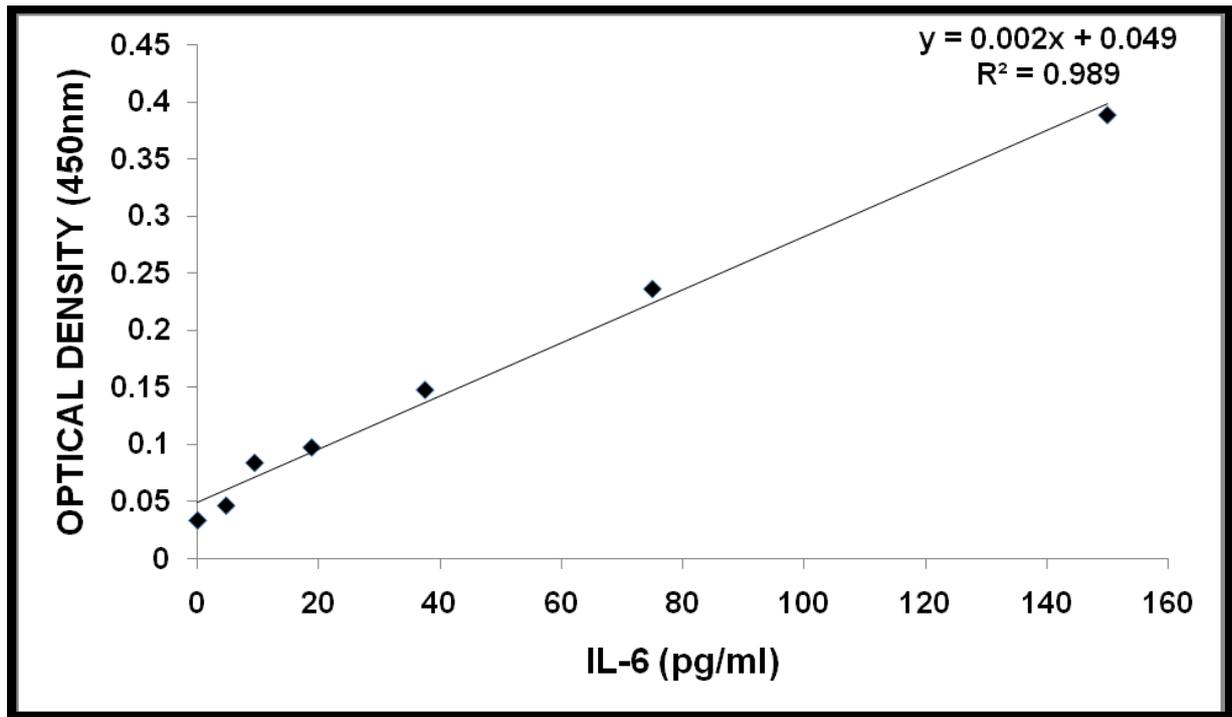


Figure 7 Standard curve derived from the IL-6 standards

Standard IL-6 concentrations were prepared and used to create the standard curve above. The curve was extrapolated and the equation ($y=0.002x + 0.049$) used to determine the serum IL-6 concentrations for each control and CAD patient.

APPENDIX 8

TaqMan[®] SNP Genotyping Assay

Following the manufacturer's protocol, TaqMan[®] SNP Predesigned Genotyping Assay (Life Technologies, Cat #4351379) was used to genotype all subjects for SIRT1 rs1467568 and rs7895833.

The TaqMan Predesigned Genotyping Assay contains two primers for amplifying the sequence of interest and two TaqMan[®] minor-groove binding (MGB) probes for detecting alleles. The presence of two probe pairs in each reaction allows genotyping of the two possible variant alleles at the SNP site in a DNA target sequence. The genotyping assay determines the presence or absence of a SNP based on the change in fluorescence of the dyes associated with the probes. The TaqMan[®] MGB Probes consist of target-specific oligonucleotides with a reporter dye at the 5' end of each probe: One VIC[®]-labelled probe to detect Allele 1 sequence (A-allele in the case of rs1467568 and rs7895833) and one FAM[™]-labelled probe to detect Allele 2 sequence (G-allele in the case of rs1467568 and rs7895833). Fluorescence signals for both dyes indicate heterozygosity for allele 1-allele 2 (AG).

A final reaction mixture consisted of 40× TaqMan[®] Predesigned Genotyping Assay, 2× TaqMan[®] Genotyping Master Mix, nuclease-free water, and a 10ng genomic DNA template. The experiment was done using the Applied Biosystems[®] ViiA[™] 7 Real-Time PCR System.

APPENDIX 9

Acceptance letter for Manuscript - Ramkaran P., Phulukdaree A., Khan S., Moodley D., and Chuturgoon A. A. (2015) Sirtuin 1 rs1467568 and rs7895833 in South African Indians with Early-onset Coronary Artery Disease. CardioVascular Journal of Africa (*In press*, October 2015)

(Manuscript number: CVJSA-D-15-00055)

Anil Chuturgoon

From: em.cvjsa.0.46cb23.62b35a20@editorialmanager.com on behalf of CardioVascular Journal of Africa <em@editorialmanager.com>
Sent: 28 October 2015 12:53 PM
To: Anil Chuturgoon
Subject: Your Submission

Ref.: Ms. No. CVJSA-D-15-00055R1
Sirtuin 1 rs1467568 and rs7895833 in South African Indians with Early-onset Coronary Artery Disease CardioVascular Journal of Africa

Dear Professor Chuturgoon,

We wish to inform you that your manuscript, Sirtuin 1 rs1467568 and rs7895833 in South African Indians with Early-onset Coronary Artery Disease, has been accepted for publication in CardioVascular Journal of Africa.

Unfortunately, we are unable to tell you which Volume and on what pages at present as we rely on our set-out design & publishers for that information.

You will be contacted by our Production Editor when your article is ready for final proof reading before publication.

Please note that the Cardiovascular Journal of Africa has launched an online First Facility with full text availability via PubMed and the Journal's website which is accessible via Google and other search engines (www.cvja.co.za). This facility is also known internationally as E-publication, ahead of print and offers authors the opportunity to publish their research articles sooner for an international audience.

Articles published online with CVJA will be published with their unique DOI numbers, which ensures that the article can be cited using the date of the manuscript's first online posting and its DOI number. DOI's provided a persistent, permanent way to identify manuscripts in an electronic environment and are generated via our Editorial Management system and in accordance with the policy of the DOI foundation (www.doi.org).

Should you wish to make use of the early publication you can logon to www.cvja.co.za and request and pay online. The CVJA has had to implement an administration fee per article if you want to make use of this option.

We thank you for your interest and support in the Journal and we will be interested in any other material you may feel you would like to submit in this field.

Thank you for submitting your work to this journal.

With kind regards

Pat J Commerford, MBChB FCP SA FACC
Editor-in-Chief
CardioVascular Journal of Africa

Comments from the Editors and Reviewers:

Reviewer #2: The changes and explanations of the manuscript are satisfying and i wish you further success on your job. Congratulations.

Reviewer #3: Thank you - the revisions were noted

1