

# The Genetics of Anthracycline-Induced Cardiotoxicity in Cancer Patients



**By**

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## Declaration

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## Abstract

**INTRODUCTION:** Breast cancer makes up 25% of all cancers diagnosed worldwide. Despite an increasing yearly incidence, there has been a significant decrease in mortality owing to early diagnosis and advances in treatment. Anthracycline-based chemotherapy is a relatively low cost yet highly effective anti-cancer treatment, increasing survival from 30% to >80%, presently. However, treatment efficacy is marred by the increased risk of anthracycline-induced cardiotoxicity (ACT) – estimated at 10-26%. Internationally, there has been evidence of ACT having a genetic basis.

Currently in South Africa, there is little information on ACT in cancer patients and survivors, and no information on the genetic basis of this phenomenon. Our recruitment sites in Cape Town - Groote Schuur Hospital (GSH) and Tygerberg Hospital (TBH), routinely treat hundreds of patients, notably with breast cancer, with anthracycline-based therapy every year, and provided the environment to assess ACT, as well as genetic factors which may influence this adverse drug reaction. Left ventricular ejection fraction (LVEF) acts as a surrogate measure of cardiac function in the public health-care setting.

**OBJECTIVES:** To provide insight into the clinical management of breast cancer patients on anthracycline-based treatment with a focus on the prevalence of ACT. To provide an index of genetic susceptibility to ACT and potentially contribute to a personalized medicine approach for a genetically diverse population.

**METHODOLOGY:** In the retrospective part of the study, the clinical records of cancer patients treated with anthracyclines from 2011- 2016 at the Oncology Clinic at GSH were analysed. Clinical co-morbidities such as hypertension, diabetes, pre-existing cardiac disease and smoking as well as type and dose of anthracyclines, cardiac function and patient status were assessed.

In the prospective study, breast cancer patients treated with anthracyclines, with a pre and post-treatment LVEF measure were recruited at GSH and TBH from 2013 to 2016. Patients were consented for access to both clinical information and biological material. Demographics, clinical risk factors and chemotherapeutic regimen data were analysed. LVEF, biomarkers and clinical status were also assessed in terms of reflecting ACT. In some instances certain clinical information was not available (i.e. LVEF) and out of necessity, a statistical correlation model or classifier was created in order to use available clinical data to derive missing clinical measures. Patients' DNA were analysed for seven genetic variants in the following six genes *ABCC1* (*rs246221*); *ABCC2* (*rs17222723*; *rs8187710*); *HNMT* (*rs17583889*); *NCF4* (*rs1883112*); *RAC2* (*rs13058338*) and *RARG* (*rs2229774*), and tested for correlation with clinical status and cardiac injury. Finally, a corollary study was conducted on a subset of patients in an attempt to determine whether cardiac biomarkers may be more sensitive measures of cardiotoxicity.

**RESULTS & DISCUSSION:** In the retrospective cohort (n=402) 19.7% of patients showed diminished cardiac function. Logistic regression showed that the following predictors: type of first line chemotherapy, and total dose significantly contributed to the ACT phenotype as measured by change in LVEF.

In the prospective patients (n=272), 14% were affected with ACT, with an increased likelihood of cardiotoxicity in the Indigenous African population. Logistic regression showed that both total anthracycline dose and change in LVEF were predictive of ACT. In the association study of prospective patients, only the *RARG rs2229774* variant was significantly associated with patient ACT status (p=0.049, Chi-Square Test).

Forty-two patients were assessed for the  $\beta$ -Natriuretic Peptide (BNP) biomarker and showed limited utility in correlating clinical status and/or LVEF decrease in all patients except Indigenous Africans indicating potential increased susceptibility of population group to ACT.

LVEF was found to be unreliable as significant LVEF decreases did not always correlate with cardiac impairment and vice-versa. Changes in routine clinical patient management and overburdening of the nuclear medicine department also translated to only one LVEF measure being obtained in some instances. The statistically derived classifier for missing indicators of heart function was useful, but will require refinement.

**CONCLUSIONS AND RECOMMENDATIONS:** Despite the inability of genotype as a predictor of ACT in this study, the increased susceptibility in the Indigenous African population to ACT as well as increased BNP levels after chemotherapy requires a closer look. The interrogation of Indigenous African patient genomes for novel variants of susceptibility to ACT are recommended; this requires building up of a substantial cohort from this population group, which would likely require collaboration with health care institutions in one of the other provinces of South Africa e.g. Eastern Cape, KwaZulu-Natal and/or Gauteng. Both this study and literature recommend the need for clinical trials for new and existing drugs on local African populations for both safety and efficacy.

Furthermore, the BNP biomarker may be better suited to the prediction of irreversible cardiac damage rather than early cardiotoxicity. Troponin, released in response to cardiomyocyte death, may be a more sensitive biomarker in predicting ACT. Similarly, the inherent variability and lack of sensitivity of LVEF as a measure of cardiac function warrants the consideration of alternatives such as echocardiography or tissue-doppler imaging.

Findings derived from this study indicate the need for refined patient management of ACT in a South African population to potentially allow for treatment with minimised risk and event-free breast cancer survival.

## Acknowledgements

“Courtesies of a small and trivial character are the ones which strike deepest in the grateful and appreciating heart.” – Henry Clay

While this PhD may have my name on it, it would have not been possible without the kindness and selfless contribution of others – for which I am both immeasurably and eternally grateful.

Firstly, to my parents Ravi and Shereen, no words can express the gratitude I have to both of you for the life you have blessed me with. The hard work and sacrifice undertaken and the motivation and love that you have given has resulted in a life with endless possibilities and for that I thank you...I only hope that I can one day bestow these same kindnesses on both of you for there is no one more deserving. I love you both so much.

Della and Tash, my lovely sisters – one here and one in heaven, thank you for always being your big sister’s greatest supporters. Della, thank you for your generosity of spirit and being the most fun person I know – being with you is like a holiday and I love you for it.

Michael, my love – in the journey of the PhD, I never expected to find you and I am so grateful that I did. Not only have you given me greater resilience in pursuing my dreams but you have inadvertently brought out the best version of me and for that I thank you.

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			}		= 0.81LVEF pre + 0.24	4		
				+3.52 - 0.05LVEF pre		= 0.81LVEF pre + 10.01	0	EC
						= 0.81LVEF pre + 5.07	2	
						= 0.81LVEF pre + 4.65	3	
						= 0.81LVEF pre + 0.45	4	
				+9.08 - 0.14LVEF pre		= 0.72LVEF pre + 15.57	0	FEC
						= 0.72LVEF pre + 10.63	2	
						= 0.72LVEF pre + 10.21	3	
						= 0.72LVEF pre + 6.01	4	
						= 0.86LVEF pre + 6.49	0	Other
						= 0.86LVEF pre + 1.55	2	
						= 0.86LVEF pre + 1.13	3	
						= 0.86LVEF pre - 3.07	4	

\*Grey font indicates little data and should be ignored.

Logistic regression was then performed with patient clinical status as the outcome and severity score and/or classifier and additional variables listed in Table 29, as the predictors.

**Table 29: Logistic Regression Coefficients used to determine Odds Ratios of covariates for Patient Clinical Status**

Covariates	Test Statistic	Odds Ratio <sup>^</sup>	p-value
		1.00	
<b>Age at Diagnosis</b>	0.019	(0.98-1.02)	0.985
<b>Gender- Male</b>	-2.245	(0.03-0.81)	0.025*
	-----	-----	-----
<b>Population Group_CA (ref)</b>	-0.370	(0.23-2.25)	0.711
<b>Population Group_MA</b>		0.81	
<b>Population Group_IA</b>	-0.369	(0.21-2.43)	0.712
<b>Hypertension</b>	0.063	(0.62-1.69)	0.950
<b>Diabetes Mellitus</b>	-0.722	(0.41-1.58)	0.470
<b>Pre-existing Cardiac Disease</b>	-0.491	(0.29-2.42)	0.623
<b>Smoking Status</b>	0.666	(0.71-2.09)	0.506
<b>First line Chemotherapy</b>	2.587	(1.17-3.20)	0.009*
<b>Total doxorubicin dose</b>	5.175	(1.005-1.01)	2.28e <sup>-07*</sup>
<b>Total epirubicin dose</b>	5.857	(1.003-1.005)	4.72e <sup>-09*</sup>
<b>Total anthracycline dose</b>	2.127	(1.000-1.003)	0.003*
<b>Change in LVEF</b>	8.98	(1.48-1.83)	<2e <sup>-16*</sup>
<b>Score (0-4)</b>	-0.003	1.88 <sup>+</sup>	0.998

\*statistically significant with a p-value threshold of <0.05; <sup>^</sup>univariate odds ratios with 95% CI; \*sample size too small for CI

The logistic regression analyses indicate that patients on first-line chemotherapy had nearly twice the odds of developing the outcome (OR=1.94, 95%CI, p=0.009). HPT has an OR of 1 indicating no association, while smoking has an OR of 1.2, this is not significant. Only those predictors with a statistically significant p-value were

considered for the multivariable model. Predictor variables were computed in multiple combinations and only the change in LVEF remained statistically significant when computed with other variables (p value=0.000164, OR=1.7).

## **4.2 Prospective Cohort Analysis**

### **4.2.1 Recruitment**

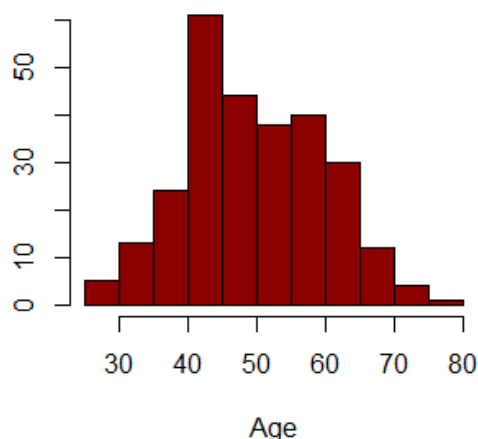
Of the 275 patients originally recruited in the prospective study, three were removed for various reasons, including a change in treatment regimen, a cerebrovascular accident (CVA/stroke) and the development of herpes zoster. One patient was recruited in the cardiology clinic after suffering a myocardial infarction due to chemotherapy – only the post-chemotherapy sample was procured.

Patients deemed to have sufficient chemotherapy for quantifiable effect, despite regimen switches or incomplete regimen, were extensively interrogated. Thirty-seven patients had only one sample procured (pre-chemotherapy). Three patients deceased before a second sample could be procured. Seven patients either discontinued or switched chemotherapeutic regimen. Twenty-seven patients were lost to follow-up despite the majority of them completing chemotherapy. After completion of the study, 25 patients (9.2%) were deceased due to cancer progression, CF or treatment-related mortality. Seventeen of the deceased patients (68%) were from GSH and eight (32%) were from TBH. Ultimately, 272 of the original 275 patients were genotyped and analysed.

### **4.2.2 Procurement of Clinical Information from Patient Folders**

The median age of the prospective patient cohort was 50 years (range 25 - 77 years). This was comparable to the retrospective cohort (median age: 51 years, and range 27 to 77 years). The Shapiro-Wilk test showed that age of the prospective cohort was normally distributed ( $W=0.99222$ ,  $p\text{-value}=0.1641$ ). IA patients were diagnosed at a median age of 44 years compared to their MA (51 years) and CA (59 years) counterparts. This was very similar to the data from the retrospective cohort for IA patients who had a median age at diagnosis of 44 years, compared to their MA (52 years) counterparts. The prospective cohort showed that the CA patients were appreciably older at diagnosis (59 years) than both the IA and MA groups

The histogram below (Fig.23) illustrates the age at diagnosis of patients in the prospectively analysed cohort.



**Figure 23: Histogram of the age at diagnosis of patients in the prospective cohort (n=272)**

All patients recruited in the study were female, diagnosed with breast cancer and anthracycline-based treatment naïve. The average BSA, used to determine accurate treatment dose, was higher than the “normal” at 1.6m<sup>2</sup> for all population groups and to a greater degree for both CA (1.84) and IA (1.81) patients. Table 30 outlines the demographic characteristics of the patients in the cohort in greater detail.

**Table 30: Demographic characteristics of patients receiving anthracycline-based chemotherapy in the prospective cohort**

	MA (n=215)	IA (n=47)	CA (n=10)	Combined (n=272)
<b>Sex (Female:Male)</b>	215 : 0	47 : 0	10 : 0	272 : 0
<b>Median Age at Diagnosis (years)</b>	51	44	59	50
<b>Body Surface Area*</b>	1.74	1.81	1.84	1.76
<b>HIV Status (Negative: Positive: Unk)</b>	193 : 5 : 17 (0.90/0.02/0.08)	27 : 20 : 0 (0.57/0.43/0)	10 : 0 : 0 (1/0/0)	230 : 25 : 17 (0.85/0.09/0.06)
<b>Tobacco Smoking (No: Yes: Unknown)</b>	122 : 77 : 16 (0.57/0.36/0.07)	41 : 5 : 1 (0.87/0.11/0.02)	6 : 3 : 1 (0.60/0.30/0.1)	169 : 85 : 18 (0.62/0.31/0.07)
<b>Hypertension (No: Yes: Unknown)</b>	116 : 99 : 0 (0.54/0.46/0)	33 : 14 : 0 (0.70/0.30/0)	4 : 6 : 0 (0.40/0.60/0)	153 : 119 : 0 (0.57/0.43/0)
<b>Diabetes (No: Yes: Unknown)</b>	190 : 25 : 0 (0.88/0.12/0)	46 : 1 : 0 (0.98/0.02/0)	9 : 1 : 0 (0.90/0.10/0)	245 : 27 : 0 (0.90/0.10/0)
<b>Cardiac Disease (No: Yes: Unknown)</b>	200 : 14 : 1 (0.93/0.065/0.005)	41 : 6 : 0 (0.87/0.13/0)	10 : 0 : 0 (1.00/0/0)	251 : 20 : 1 (0.92/0.07/0.004)

\* BSA in females is considered normal at approx. 1.6m<sup>2</sup>

The majority of patients in the cohort were HIV negative (85%); however further analysis revealed that a significant number of IA patients were HIV positive (43%) compared to MA patients (2%) and Caucasian patients (0%). Conversely in terms of smoking, hypertension and diabetes, IA patients self-reported the lowest incidence – with smokers (11%), hypertensives (30%) and diabetics (2%) compared to MA patients – smokers (36%), hypertensives (46%) and diabetics (12%) and Caucasian patients – smokers (30%), hypertensives (60%) and diabetics (10%).

Despite the majority of patients not having any pre-existing cardiac disease (92%), early measures of cardiac function before administration of chemotherapy revealed minor abnormalities in approximately 5% of patients. IA patients had the highest incidence of pre-existing cardiac disease (13%).

Table 31 shows both the type and stage of breast cancer of all the patients in the cohort.

**Table 31: Type and Stage of Cancer of Patients in the Prospective Cohort (n=272)**

<u>Type of Cancer</u>	<u>Stage of Cancer</u>									
	<u>IA</u>	<u>IB</u>	<u>IIA</u>	<u>IIB</u>	<u>IIIA</u>	<u>IIIB</u>	<u>IIIC</u>	<u>IV</u>	<u>Unknown</u>	<u>Total</u>
<b>DCIS or LCIS</b>	2	0	7	9	7	9	1	0	0	35
<b>IDC</b>	8	2	45	53	30	42	9	2	2	193
<b>ILC</b>	0	0	3	4	2	4	0	1	0	14
<b>Inflammatory</b>	0	0	0	0	0	2	0	0	0	2
<b>Invasive</b>	0	0	3	3	2	2	0	0	0	10
<b>BC NOS*</b>	1	0	1	0	1	3	0	0	0	6
<b>Metastatic</b>	0	0	1	0	2	3	0	5	1	12
<b>Total</b>	11	2	60	69	44	65	10	8	3	272

\*BC NOS – Breast Cancer, not otherwise specified

As with the retrospective cohort, the most frequently diagnosed type of cancer was IDC (71% of the entire cohort) with the majority of these patients diagnosed at stage IIB (27% of all IDC patients). Inflammatory breast cancer was diagnosed most infrequently at only 0.7% of the cohort, consistent with the rare presentation of this subtype in the general population (Table 31).

Fifteen patients who were assessed at baseline before the administration of chemotherapy were found to have incidental cardiac findings (Table 32). One patient had an inconclusive LVEF measure.

Of the 15 patients who were found to have some measure of cardiac impairment/dysfunction before the start of chemotherapy – 20% were IA and 80% of

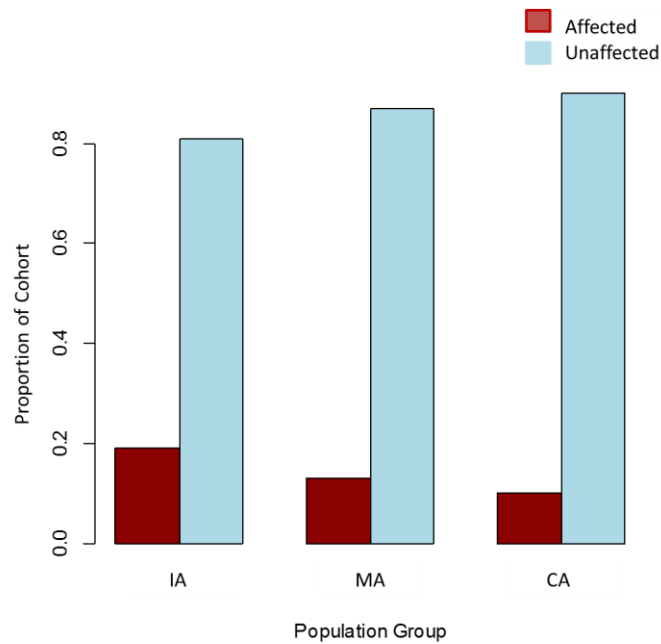
were MA, approximately reflecting the relative distribution of the ethnicities in the total cohort.

**Table 32: Incidental cardiac findings derived from routine assessment of baseline cardiac function before commencement of anthracycline-based chemotherapy contrasted with baseline LVEF**

<u>Cardiac Incidental Findings</u>	<u>LVEF (%)</u>	<u>Population Group</u>
Rhythm abnormality	52	IA
Mild cardiomegaly	60	IA
Rhythm abnormality	-	MA
Aortic Regurgitation	60	MA
Impaired relaxation indicated by diastolic filling pattern	55	MA
Left ventricular hypertrophy	70	MA
Cardiomegaly with pericardial effusion	54	MA
Left ventricular hypertrophy; elevated left atrial filling pressures; mildly dilated left atrium	60	MA
Wall motion impairment	55	MA
Left ventricular hypertrophy with normal diastolic filling pattern	55	IA
Impaired relaxation of left ventricle	55	MA
Impaired relaxation with elevated left atrial filling pressures	55	MA
Aortic regurgitation; impaired relaxation of left ventricle; early mild pulmonary hypertension	60	MA
Tricuspid regurgitation	60	MA
Left ventricular hypertrophy	55	MA

### 4.2.3 Statistical Analysis

Patient status was deemed either affected or unaffected with signs or symptoms of ACT. There were 38 affected (14% of the cohort) and 234 unaffected patients at the conclusion of chemotherapy in the prospective cohort. However this clinical status could be considered provisional as unaffected patients may have subclinical damage that may manifest later.



**Figure 24: Clinical status (Cardiac impairment/dysfunction: affected vs unaffected) of Patients stratified by Population Group in Prospective Cohort**

Clinical status (Cardiac impairment/dysfunction: affected vs unaffected) differed between population groups ( $p=0.5116$ ), where 19% of IA patients were classified as affected compared to 13% of MA and 10% of CA indicating that IAs may have increased sensitivity to ACT (Fig.24).

The score or classifier was established to allow for patients with missing post-treatment LVEF ( $n=185$ ) to be assessed in cardiac terms with 0 being 'normal' and 4 being 'most severely affected'. The differences between the population groups was found to be statistically significant (Chi-square,  $p=0.036$ ).

**Table 33: Logistic Regression Co-efficients used to determine Odds Ratios using Clinical-based Covariates**

Covariates	Test Statistic	Odds Ratio <sup>^</sup>	p-value
Age	-0.610	0.99 (0.96-1.02)	0.542
Population Group_IA (ref)	-----	-----	-----
Population Group_MA	1.085	1.58 (0.66-3.5)	0.278
Population Group_CA	0.677	2.13 (0.33-41.9)	0.498
Hypertension	-1.184	0.66 (0.33-1.31)	0.236
Diabetes Mellitus	0.450	1.33 (0.44-5.82)	0.653
Pre-existing Cardiac Disease	-0.131	0.92 (0.29-4.08)	0.896
Smoking Status	0.144	1.06 (0.51-2.29)	0.886
Change in LVEF	3.891	1.65 (1.34-2.25)	9.99e <sup>-05*</sup>
Total Dose of Anthracycline-Based Chemotherapy	2.400	1.002 (1.0004-1.004)	0.0164*
Change in LVEF and Total Dose	3.769	1.704 (1.36-2.39)	0.000164*

\*statistically significant; <sup>^</sup>odds ratios with 95% CI

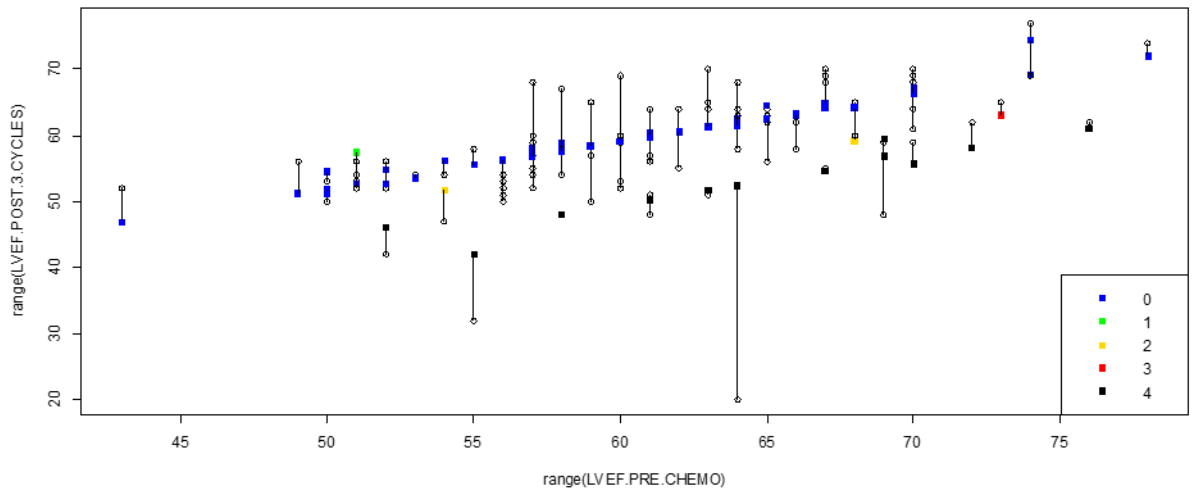
Gender was not used as a covariate due to all the patients in the prospective cohort being female.

Therefore, based on both the OR and p-values, the change in LVEF and total dose of anthracycline-based chemotherapy significantly increased the likelihood of cardiotoxicity.

The change in LVEF as a predictor of the outcome of ACT, meant that missing LVEF measures needed to be addressed. A simple model was established whereby patients with missing variables (i.e. LVEF post-chemo) could be estimated using the clinical classifier/score developed during this study as well as population group and type of first-line chemotherapy regimen.

Retrospective data (n=402) where two LVEF measures, together with qualitative and quantitative cardiac data, where available, (and scored from 0-4) was used to create a formula that was validated on prospective patients (n=87) with both pre and post-chemo LVEF measures. The formula was then applied to prospective patients (n=185) with only one LVEF measure (pre-chemo) to predict post-chemo LVEF

measures. The difference between actual and predicted post-chemo LVEF measures is illustrated in Figure 25.



**Figure 25: Difference between actual and predicted post-chemo LVEF measures in prospective patients (n=87) – Observed LVEF measures are represented as open circles with predicted values colour-coded according to severity score**

A cut-off of 4% was utilized as literature indicates that measurements of LVEF accounting for both inter-operator variability and repeat testing on the same patient are acceptable if in the range of 2-4%<sup>134</sup>.

Therefore the proposed model indicates an 82% accuracy rate in predicting post-chemo LVEF and can be used for missing variables (LVEF measure) for the rest of the prospective patient cohort.

### 4.3 Patient Sample

#### 4.3.1 DNA isolation

DNA was isolated from 275 patient samples using the Promega Maxwell® 16 DNA Purification Kit and the salting out method. The Promega Maxwell® 16 DNA Purification Kit had a quick turnaround time but produced low yields of isolated DNA.

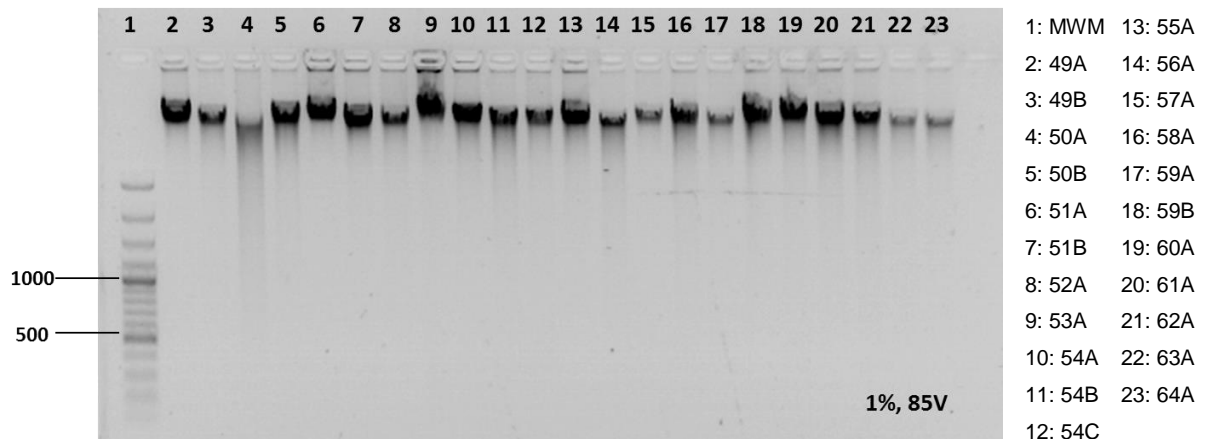
DNA was successfully isolated from 274 patients, only one patient had insufficient white blood cells for the isolation of DNA. All patient DNA, after being quantified using spectrophotometry, was stored at -20°C until required for downstream applications (Appendix H, Table 1). The majority of patient samples taken after chemotherapy were stored as buffy coats and isolated as needed.

#### 4.3.1.1 Spectrophotometry for quantitation of DNA

All DNA samples were spectrophotometrically quantified before PCR (Appendix I, Fig. 1) and a total of 86 of the 274 patient DNA samples had concentrations less than 100ng/ul – the optimal concentration for PCR. This was noted in anticipation of potential failure to amplify.

#### 4.3.1.2 Assessment of DNA Integrity

The integrity of the patient DNA was assessed using agarose gel electrophoresis (Fig.26). Low molecular weight DNA or degraded DNA appeared as a smear on the gel whereas intact DNA appeared as a sharp band proximal to the loading well.



**Figure 26: Assessment of DNA Integrity using agarose gel electrophoresis. Patient DNA samples were electrophoresed at 85V for 2 hours on a 1% (w/v) agarose gel (Seakem® LE Agarose, Lonza, USA) stained with SYBR®Safe (Life Technologies, USA) and visualized under UV light. The molecular weight marker (MWM) used was the GeneRuler™ 100bp Plus DNA Ladder (ThermoScientific, USA).**

This DNA integrity gel (Fig.26) indicates that the majority of the patient DNA samples are utilisable for downstream applications. However the smeared band for sample 50A (lane 4) and the feint bands for samples 63A and 64A (lanes 22 and 23) may be partially degraded and/or of relatively low concentration. Spectrophotometry shows that sample 50A (lane 4) has a sufficiently high concentration (452.16 ng/ul) therefore is likely to be degraded. Both samples 63A (25.1 ng/ul) and 64A (29.8 ng/ul) have low concentrations which explains the feint band visualized on the gel.

Cumulatively, 22 samples had either very low concentrations and appeared on the gel as feint bands or were partially degraded and appeared on the gel as smears. Nevertheless all 22 samples were amplified using PCR – only six samples exhibiting

partial degradation and two samples with very low concentrations were problematic to amplify and genotype.

## 4.4 Selected Genes of Interest

Genes of interest were selected using both a pathway-based<sup>83</sup> and candidate gene/marker approach based on previous studies<sup>10,12,27,34,47,106,135-137</sup>.

Initially, 19 SNPs in 15 genes described in Table 7 were selected using a candidate gene approach. However, after approximately 70 patients were genotyped, genotypes appeared to be monomorphic for some of the SNPs, therefore only seven SNPs (*ABCC1* rs246221; *ABCC2* rs17222723; *ABCC2* rs8187710; *HNMT* rs17583889; *NCF4* rs1883112, *RAC2* rs13058338 and *RARG* rs2229774) that showed both heterozygosity and association with the cardiac phenotype were further interrogated for all patient samples (Table 7).

### 4.4.1 Gene Annotation

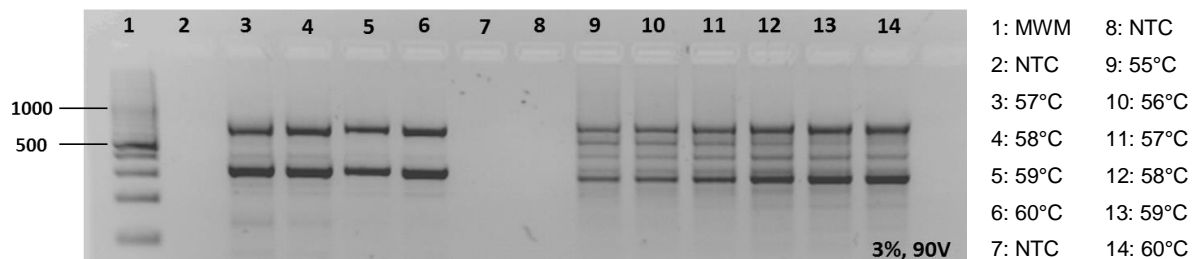
The output derived from Appendix J Figure 1, allowed for both the confirmation of the genetic location of the SNP as well as the design of primers found later in the chapter (Appendix J, Figure 1).

## 4.5 PCR

### 4.5.1 Optimisation: Singleplex and Multiplex PCR

#### 4.5.1.1 Temperature Gradient

The first PCR optimisation step was establishing a good melting temperature where there would be optimal yield of specific product.

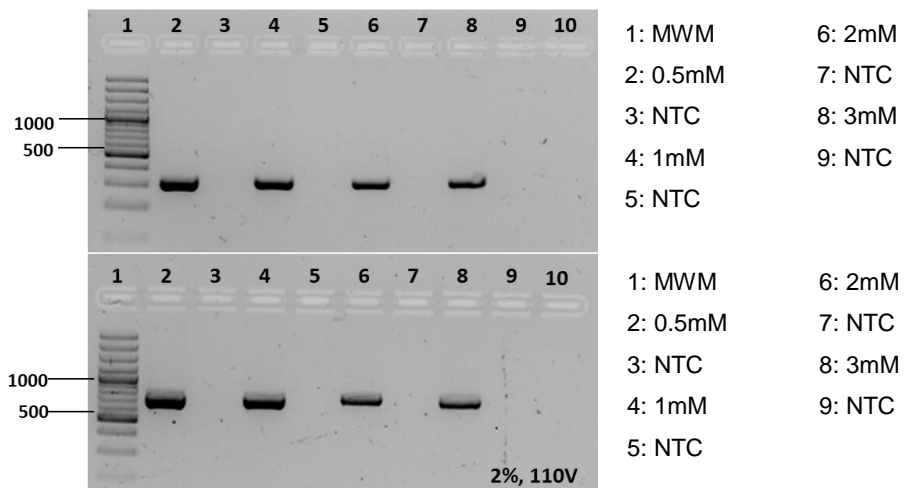


**Figure 27: Multiplex optimisation using a temperature gradient and visualized by agarose gel electrophoresis. Control DNA (C18F) was amplified for *ABCC1* rs4148350 (333bp) and *CYBA* rs4673 (680bp) [lanes 2-6] and *ACO1* rs867469 (307bp) and *ABCC2* rs17222723 (761bp) [lanes 8-14] and were electrophoresed at 90V for 2 hours on a 3% (w/v) agarose gel (Seakem® LE Agarose, Lonza, USA); MWM = GeneRuler™ 100bp Plus DNA Ladder**

The first multiplex involving the amplification of both *ABCC1* rs4148350 (333bp) and *CYBA* rs4673 (680bp) on a temperature gradient between 57-60°C showed greatest specificity and efficiency at  $T_m=60^\circ\text{C}$  (lane 6). Temperatures lower than 60°C resulted in non-specific amplification (lanes 3-5). The second multiplex involving the amplification of both *ACO1* rs867469 (307bp) and *ABCC2* rs17222723 (761bp) showed increased yield of both regions as the temperature increased, however, non-specific amplification persisted (lanes 9-14). There was no amplification in both NTCs (lanes 2 and 8) therefore contamination of the PCRs were unlikely.

#### 4.5.1.2 Magnesium Gradient

The optimisation of the concentration of magnesium to remove any non-specific amplification (Fig.28)

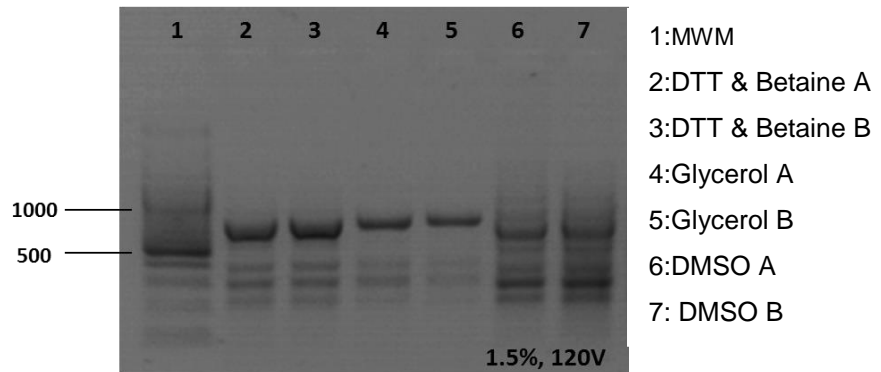


**Figure 28: Singleplex optimisation using a magnesium chloride ( $\text{MgCl}_2$ ) gradient and visualized by agarose gel electrophoresis. Control DNA (C18F) was amplified for *ABCC1* rs4148350 (333bp) [top gel] and *NCF4* rs1883112 (725bp) [bottom gel] and were electrophoresed at 110V for 1 hour on a 2% (w/v) agarose gel (Seakem® LE Agarose, Lonza, USA); MWM = GeneRuler™ 100bp Plus DNA Ladder (ThermoScientific, USA); NTC= no template control**

Amplification for both *ABCC1* rs4148350 (333bp) [top gel – lanes 2-9] and *NCF4* rs1883112 (725bp) [bottom gel – lanes 2-9] were optimised using an  $\text{MgCl}_2$  gradient with concentrations at 0.5mM (lanes 2 & 3), 1mM (lanes 4 & 5), 2mM (lanes 6 & 7) and 3mM (lanes 7 & 8). Amplification for both *ABCC1* rs4148350 (top gel) and *NCF4* rs1883112 (bottom gel) had optimum yield and specificity at an  $\text{MgCl}_2$  concentration of 1-2mM (lanes 4 & 6). NTCs for each sample showed no amplification therefore contamination was unlikely.

#### 4.5.1.3 Additives

Alterations to the annealing temperature and  $MgCl_2$  concentration did not always guarantee an optimised PCR, therefore, the addition of certain additives were utilized to improve specificity (Fig.29).



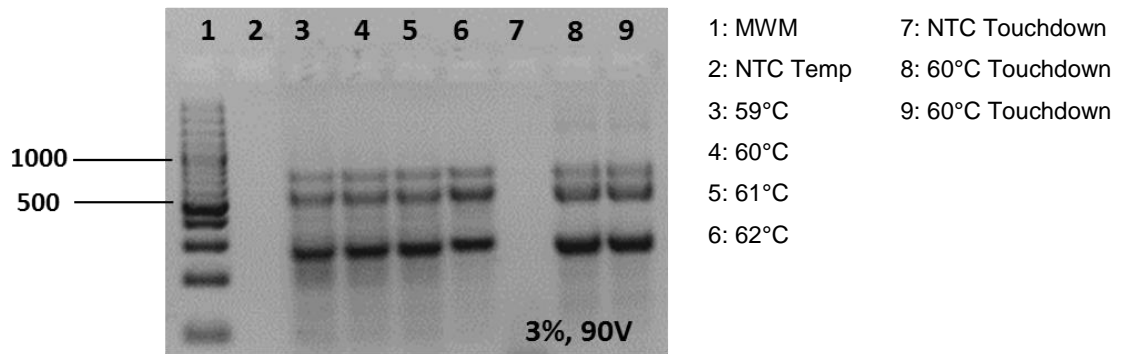
**Figure 29: Singleplex optimisation of ABCC2 rs17222723 (761bp) using additives (Dithiothreitol (DTT), Betaine, Glycerol and dimethyl sulfoxide (DMSO)) and visualized by agarose gel electrophoresis. Control DNA (C18F) was amplified for ABCC2 rs17222723 (761bp) in duplicate (A & B) and were electrophoresed at 120V for 40 minutes on a 1.5% (w/v) agarose gel (Seakem® LE Agarose, Lonza, USA); MWM = GeneRuler™ 100bp Plus DNA Ladder (ThermoScientific, USA)**

Amplification of *ABCC2* rs17222723 (761bp) showed least non-specificity with the addition of Glycerol (Lanes 4 & 5). The addition of both DTT and Betaine (Lanes 2 & 3) was less successful – while the target region was amplified at a sufficiently high yield, there were three non-specific bands. The addition of DMSO (Lanes 6 & 7) was the least successful- there were multiple bands of non-specific amplification with one of the bands having as high a yield as the target region. This challenging PCR required further optimisation whereby non-specific amplification was eliminated.

#### 4.5.1.4 Touchdown PCR

Touchdown PCR allows for the both the amplification of difficult to amplify regions as well as the elimination of non-specific products.

Previously, the addition of glycerol showed a reduction in non-specific amplification however some measure of non-specificity persisted. Touchdown PCR (Fig.30), eliminated non-specific bands and the yield of the target PCR products was high (lanes 8 & 9) compared to the temperature gradient (lanes 3-6) where non-specificity is most apparent at lower temperatures. There was no amplification in either of the two NTCs indicating the absence of contamination.

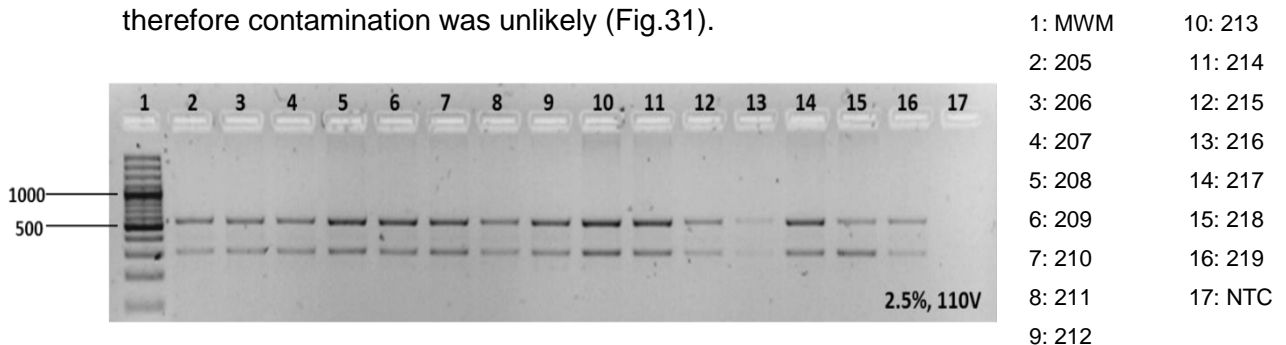


**Figure 30: Multiplex optimisation of *ACO1* rs867469 (307bp), *ABCC1* rs246221 (595bp) and *ABCC2* rs17222723 (761bp) using the additive Glycerol and comparing a temperature gradient (59°C-62°C: lanes 3-6) to touchdown PCR performed at 60°C (lanes 7-9) and visualized by agarose gel electrophoresis. Control DNA (C18F) was amplified in a multiplex PCR for *ACO1* rs867469 (307bp), *ABCC1* rs246221 (595bp) and *ABCC2* rs17222723 (761bp) and were electrophoresed at 90V for 2 hours on a 3% (w/v) agarose gel (Seakem® LE Agarose, Lonza, USA); MWM = GeneRuler™ 100bp Plus DNA Ladder (ThermoScientific, USA); NTC = no template control**

While the annealing temperature of 62°C for a standard PCR (lane 6) also showed reduced non-specific amplification, the intensity of the bands which represent amplified target regions was greater in the touchdown PCR (lanes 8-9) indicating greater yield of product and therefore greater overall efficiency. Hence the amplification of these regions was optimised with the addition of glycerol and utilizing a touchdown PCR.

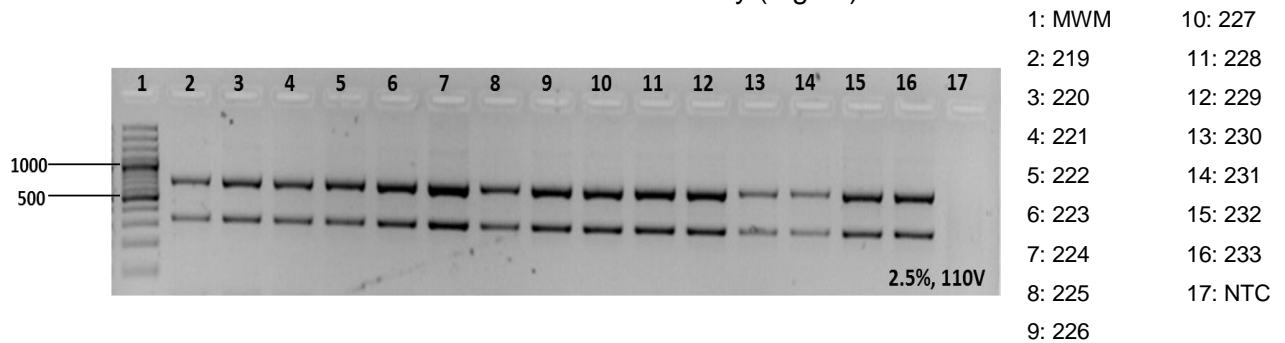
#### 4.5.2 Optimised Multiplex PCRs

All samples (lanes 2-16) amplified specifically for both target regions – *HNMT* rs17583889 (325bp) and *ABCC2* rs8187710. There was no amplification in the NTC therefore contamination was unlikely (Fig.31).



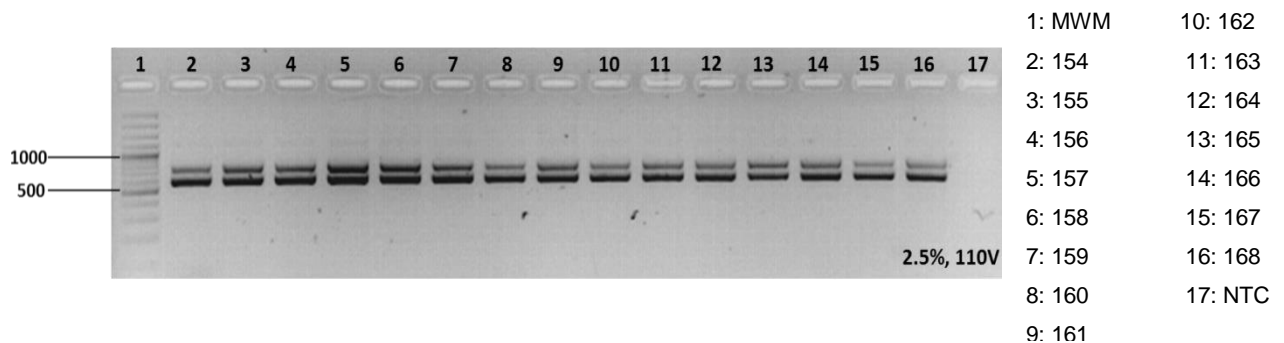
**Figure 31: Optimised multiplex PCR showing the amplification of *HNMT* rs17583889 (325bp) and *ABCC2* rs8187710 (572bp) performed at 55°C and visualized by agarose gel electrophoresis. Patient DNA was amplified in a multiplex PCR for *HNMT* rs17583889 (325bp) and *ABCC2* rs8187710 (572bp) and were electrophoresed at 110V for 1.5 hours on a 2.5% (w/v) agarose gel (Seakem® LE Agarose, Lonza, USA); MWM = GeneRuler™ 100bp Plus DNA Ladder (ThermoScientific, USA); NTC = no template control**

All samples (lanes 2-16) amplified specifically and efficiently for both target regions – *RAC2* rs13058338 (341bp) and *NCF4* rs1883112 (725bp). There was no amplification in the NTC therefore contamination was unlikely (Fig.32).



**Figure 32: Optimised multiplex PCR showing the amplification of *RAC2* rs13058338 (341bp) and *NCF4* rs1883112 (725bp) performed at 56°C and visualized by agarose gel electrophoresis. Patient DNA was amplified in a multiplex PCR for *RAC2* rs13058338 (341bp) and *NCF4* rs1883112 (725bp) and were electrophoresed at 110V for 1.5 hours on a 2.5% (w/v) agarose gel (Seakem® LE Agarose, Lonza, USA); MWM = GeneRuler™ 100bp Plus DNA Ladder (ThermoScientific, USA); NTC = no template control**

All samples (lanes 2-16) amplified specifically and efficiently for both target regions – *ABCC1* rs246221 (595bp) and *ABCC2* rs17222723 (761bp). There was no amplification in the NTC therefore contamination was unlikely (Fig.33).

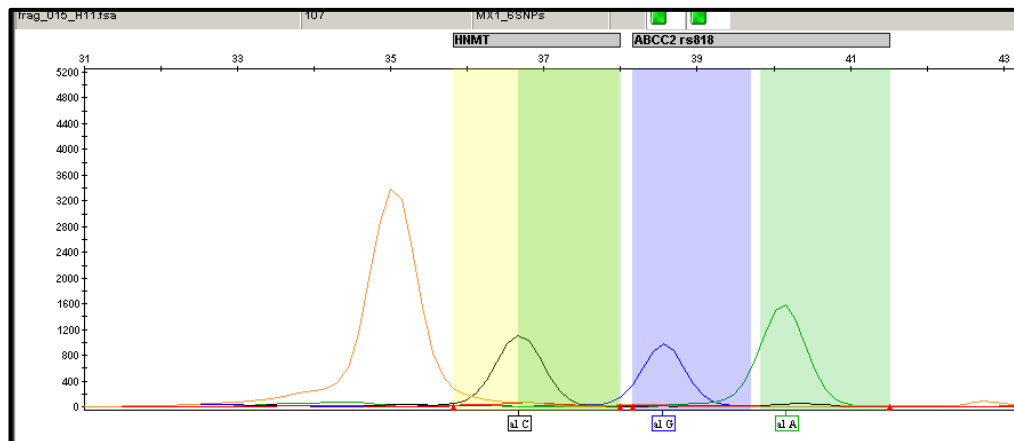


**Figure 33: Optimised multiplex PCR showing the amplification of *ABCC1* rs246221 (595bp) and *ABCC2* rs17222723 (761bp) performed using a Touchdown PCR at 60°C with the addition of glycerol and visualized by agarose gel electrophoresis. Patient DNA was amplified in a multiplex PCR for *ABCC1* rs246221 (595bp) and *ABCC2* rs17222723 (761bp) and were electrophoresed at 110V for 1.5 hours on a 2.5% (w/v) agarose gel (Seakem® LE Agarose, Lonza, USA); MWM = GeneRuler™ 100bp Plus DNA Ladder (ThermoScientific, USA); NTC = no template control**

## 4.6 Genotyping

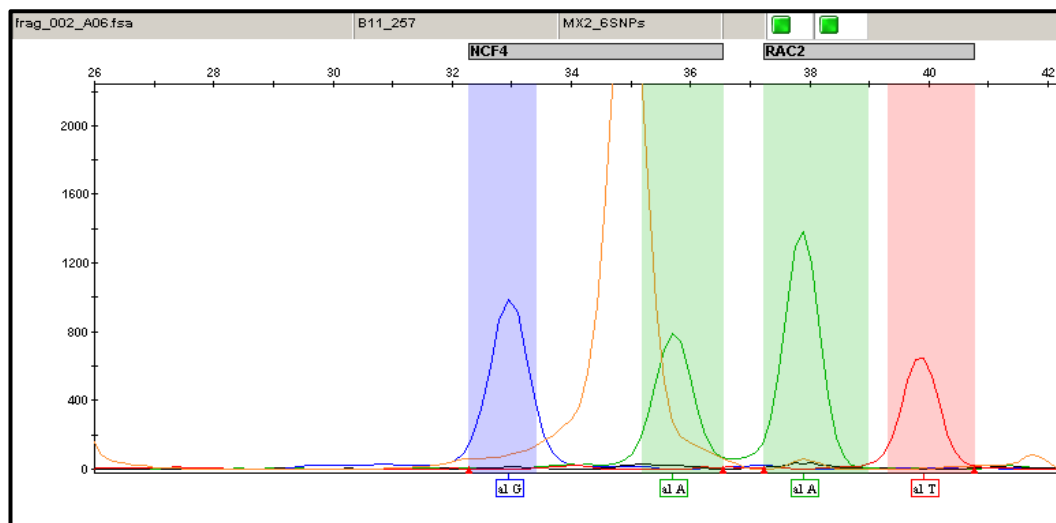
### 4.6.1 Optimisation of the SNaPshot® Multiplex System

Multiplex PCRs were optimised for genotyping using the SNaPshot® System (ThermoFisherScientific, Applied Biosystems, USA). Patient ACT 107.1NOM was genotyped for both *HNMT* rs17583889 and *ABCC2* rs8187710 and found to be a C/C homozygote and a G/A heterozygote respectively (Fig.34).



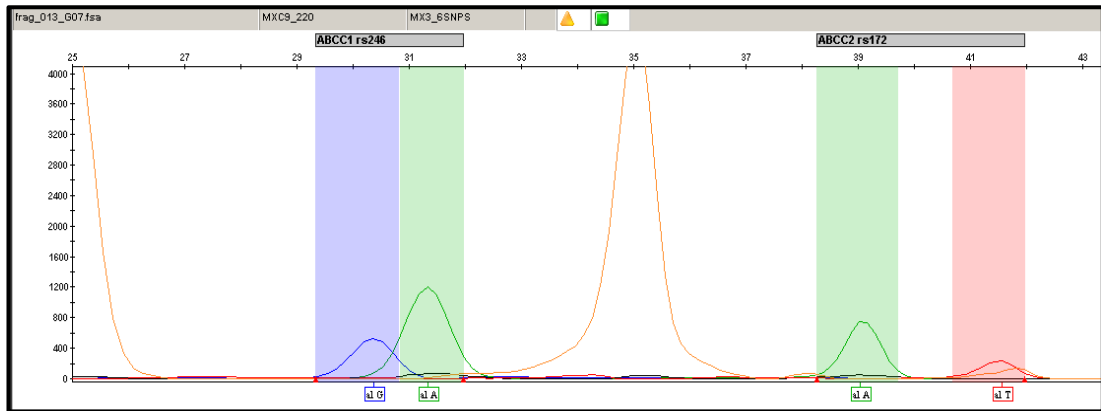
**Figure 34:** Electropherogram illustrating determination of genotypes for both *HNMT* rs17583889 (black peak C/C) and *ABCC2* rs8187710 (blue and green peaks G/A) derived from GeneMapper (version 4.1 using the ABI3130xl Genetic Analyzer (Applied Biosystems, USA). The orange peak indicates the GeneScan™ 120 LIZ® Size Standard (35 nt).

Patient ACT 257.1ANN was genotyped for both *NCF4* rs1883112 and *RAC2* rs13058338 and found to be a G/A heterozygote and an A/T heterozygote respectively (Fig.35).



**Figure 35:** Electropherogram illustrating determination of genotypes for both *NCF4* rs1883112 (blue and green peaks G/A) and *RAC2* rs13058338 (green and red peaks A/T) derived from GeneMapper (version 4.1 using the ABI3130xl Genetic Analyzer (Applied Biosystems, USA). The orange peak indicates the GeneScan™ 120 LIZ® Size Standard (35 nt)

Patient ACT 200.1KAR was genotyped for both *ABCC1* rs246221 and *ABCC2* rs1722723 and found to be a G/A heterozygote and an A/T heterozygote respectively (Fig.36).

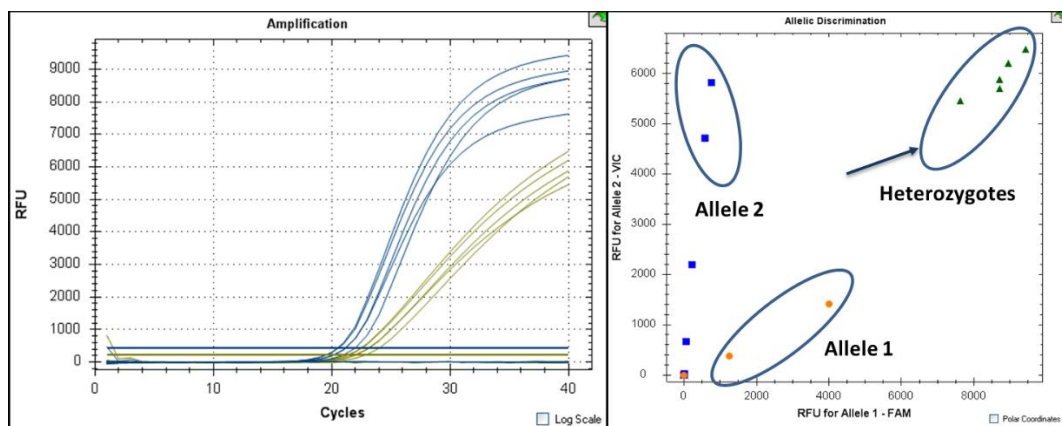


**Figure 36: Electropherogram illustrating determination of genotypes for both *ABCC1* rs246221 (blue and green peaks G/A) and *ABCC2* rs1722723 (green and red peaks A/T) derived from GeneMapper (version 4.1 using the ABI3130xl Genetic Analyzer (Applied Biosystems, USA). The orange peak indicates the GeneScan™ 120 LIZ® Size Standard (35 nt)**

#### 4.6.2 Optimisation of the TaqMan™ SNP Genotyping Assay

The concentration and integrity of the control DNA was adequate (124ng/ul) for the optimisation of the assay (Appendix H, Fig. 2). The assay was optimised by the utilization of varying concentrations of DNA to delineate the optimal volume of DNA needed for a distinct genotype to be called.

Control DNA Sample (C35F) was genotyped as a heterozygote (A/G) indicated by the blue arrow in Figure 37. The optimal volume was determined to be 2-3ul at a concentration of 100ng/ul.



**Figure 37: Quantification & Allelic Discrimination Plots generated from the CFX™ Real-Time PCR Detection System together with the TaqMan™ Genotyper Software**

### 4.6.3 Genotyping of patient samples

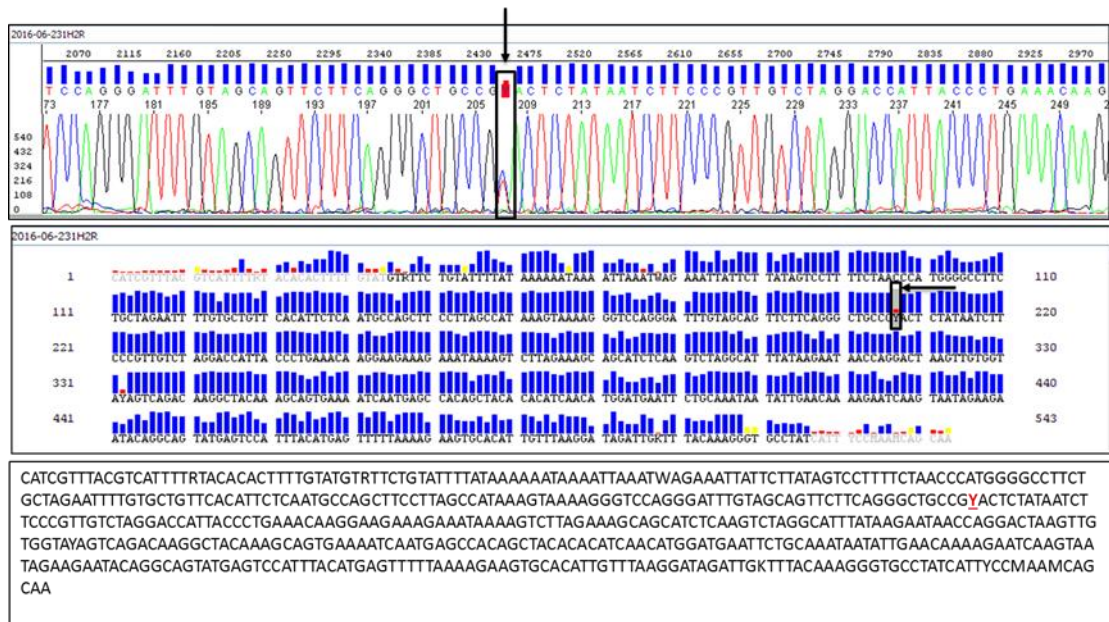
Nineteen SNPs in fifteen genes, described in Table 7, were selected using a candidate gene approach. However, only seven SNPs were interrogated for all patient samples: *ABCC1* rs246221; *ABCC2* rs17222723; *ABCC2* rs8187710; *HNMT* rs17583889; *NCF4* rs1883112, *RAC2* rs13058338 and *RARG* rs2229774.

Genotypes were derived for the following number of patients: *ABCC1* rs246221 (n=270), *ABCC2* rs17222723 (n=260), *ABCC2* rs8187710 (n=267), *HNMT* rs17583889 (n=250), *NCF4* rs1883112 (n=259), *RAC2* rs13058338 (n=271) and *RARG* rs2229774 (n=273).

### 4.6.4 Validation Sequencing

Genotyping results were validated using Sanger sequencing and 5% of the entire selection of seven SNPs were sequenced. Statistical significance is achieved when  $p < 0.05$  therefore 5% was used as a threshold for validation sequencing.

Electropherograms derived from the genetic analyser were assessed for accuracy of sequencing and to validate variants found when carrying out SNP genotyping (Fig. 38).



**Figure 38: Representation of electropherogram (top panel), sequence coverage (middle panel) and sequence (bottom panel) derived from Sanger sequencing conducted to validate SNaPshot® genotyping assay results. Patient 249.1JAN's genotyping results obtained from the SNaPshot® genotyping assay was validated using Sanger/Direct Cycle sequencing. The arrow on the top and middle panel show the location of the SNP for *ABCC2* rs8187710 (572bp), Y indicates a heterozygote (C/T)**

The genotyping primer designed for the SNaPshot® Assay for the detection of the *ABCC2* rs8187710 variant was in the forward direction with expected genotypes of GG/GA/AA however since the reverse PCR primer was found to be optimal for sequencing, the resulting electropherogram trace and sequence (Fig.38) was reverse complemented. Therefore Patient 249.1JAN's genotype according to SNaPshot® was G/A which translates to C/T which was obtained by confirmatory sequencing.

Each region of amplification and SNP was confirmed to be correct by the utilisation of the nucleotide basic local alignment search tool (BLAST) <sup>138</sup>.

The context sequence (Fig.39) was correlated to the reverse complement sequence that was already amplified (Fig.38).



**Figure 39: Representation of nucleotide blast results for *ABCC2* rs8187710 validating both the target gene and corresponding SNP**

## **4.7 Statistical Analysis**

### **4.7.1 Genotype and Allele frequencies**

Genotype counts and minor allele frequencies for each of the seven SNPs are listed in Table 34.

**Table 34: Distribution of genotypes and minor allele frequencies for seven SNPs focusing on differences in population groups**

<u>Gene Variants</u>	<u>**Genotypic Distribution</u>					<u>Minor Allele Frequencies</u>					<u>P value*</u>	<u>P value*</u>	<u>P value*</u>	
	Lit <sup>#1</sup>	All	IA	MA	Caucasian	Lit <sup>#1</sup>	All	IA	MA	Caucasian				
<b>ABCC1</b> <b>rs246221</b>	934/1013/557	80/123/67	6/19/22	68/100/45	6/4/0	0.42	0.48	0.67	0.45	0.2	0.0737	<0.0001*	0.6951 0.1275	0.0009*
<b>ABCC2</b> <b>rs17222723</b>	2321/179/4	201/54/5	34/11/1	158/42/4	9/1/0	0.04	0.12	0.14	0.12	0.05	<0.0001*	<0.0001* 0.9566	0.0352*	
<b>ABCC2</b> <b>rs8187710</b>	2181/306/17	153/98/16	19/23/4	125/74/12	9/1/0	0.07	0.24	0.34	0.23	0.05	<0.0001*	<0.0001* 0.9508	0.0285*	
<b>HNMT</b> <b>rs17583889</b>	1922/516/66	206/29/15	41/0/2	157/28/13	8/1/0	0.13	0.12	0.05	0.14	0.06	0.7682	0.0741 0.9241 0.6474	0.0152*	
<b>NCF4</b> <b>rs1883112</b>	938/1059/507	124/91/44	30/14/1	91/73/40	3/4/3	0.41	0.35	0.18	0.38	0.5	0.0106*	<0.0001* 0.3068 0.7378	0.0009*	
<b>RAC2</b> <b>rs13058338</b>	1794/91/619	200/65/6	42/5/0	152/57/5	6/3/1	0.16	0.14	0.05	0.16	0.25	0.5561	0.0192* 0.9831 0.5488	0.0113*	
<b>RARG</b> <b>rs2229774</b>	2101/367/36	217/49/5	40/7/0	168/41/5	9/1/0	0.09	0.11	0.07	0.12	0.05	0.4798	0.9043 0.0921 0.8379	0.0226*	

# = p-value derived from a chi-square test with a 95% confidence interval where the MAFs of all the SNPs under investigation were compared to the MAFs of these SNPs reported in the literature; # = p values derived from a Chi-square test with 95% confidence interval where the MAFs of each population group was compared to literature; += p values after 2-way ANOVA with 95% confidence interval to determine whether population group MAFs are significantly different to each other

\*\*Genotypic Distribution: Ratios indicate homozygous major/ heterozygous/ homozygous minor allele

Table 34 shows that the *ABCC2* rs17222723, *ABCC2* rs8187710 and *NCF4* rs1883112 polymorphisms had minor allele frequencies (MAF) in our patient cohort that were statistically different from literature. The MAFs of all the SNPs were statistically different when compared between the three population groups with the MAFs for IAs for the following SNPs: *ABCC1* rs246221, *ABCC2* rs17222723, *ABCC2* rs8187710, *NCF4* rs1883112 and *RAC2* rs13058338 all being significantly different from the literature. Only *ABCC2* rs17222723 and *ABCC2* rs8187710 in the MA population were statistically significant from literature with none of the variants in the CA cohort being statistically different to literature.

Hardy-Weinberg calculations indicated that both *HNMT* rs17583889 and *NCF4* rs1883112 are not in Hardy-Weinberg equilibrium whereas all the other SNPs that were included in the final analysis were in HWE (Table 35).

**Table 35: Patient population allele frequencies and Pearson's Chi-Square test for HWE**

Gene Variant	Alleles	Allele Frequency	HWE $\chi^2$ value	p-value*
<b><i>ABCC1</i> rs246221</b>	A	0.52	2.61	0.12
	G	0.48		
<b><i>ABCC2</i> rs17222723</b>	A	0.88	1.01	0.4
	T	0.12		
<b><i>ABCC2</i> rs8187710</b>	G	0.76	0.01	1
	A	0.24		
<b><i>HNMT</i> rs17583889</b>	C	0.88	36.86	9.99e <sup>-05</sup>
	A	0.12		
<b><i>NCF4</i> rs1883112</b>	G	0.65	12.71	4e <sup>-04</sup>
	A	0.35		
<b><i>RAC2</i> rs13058338</b>	A	0.86	0.03	1
	T	0.14		
<b><i>RARG</i> rs2229774</b>	G	0.89	1.25	0.34
	A	0.11		

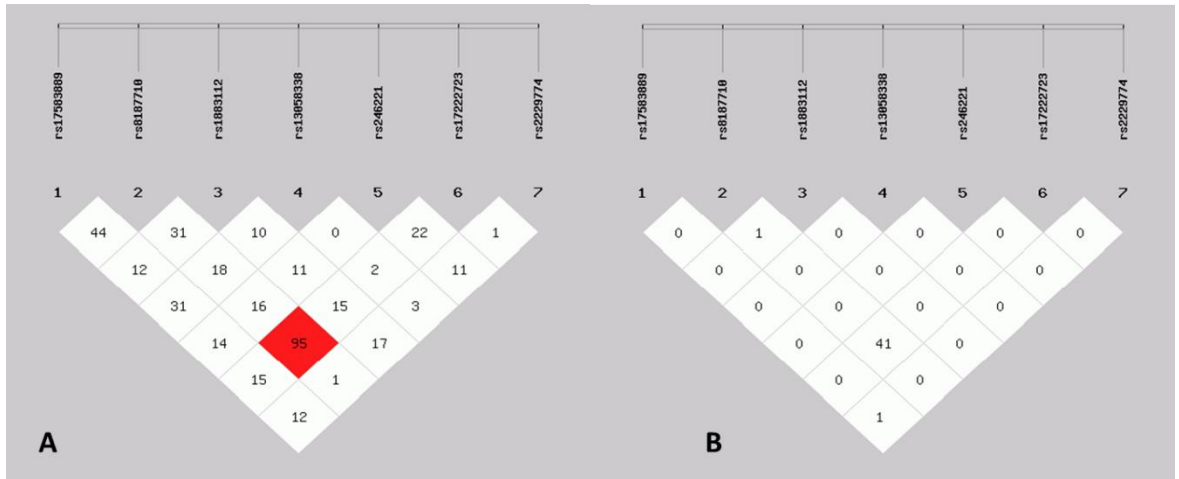
\*simulated p-value based on 1000 replicates using R

Both the  $D'$  and  $r^2$  values were used to interpret LD – *ABCC2* rs8187710 and *ABCC2* rs17222723 indicate that there was very little to no recombination between markers ( $D'=0.954$ ) and that they were in fairly strong LD ( $r^2=0.414$ ). The other variants analysed showed some degree of recombination and very weak LD (Table 36).

**Table 36: Testing for LD of genetic variants using R<sup>101</sup> and the SHESIS Program**  
132

Pairwise LD			LD Test			
Variant 1	Variant 2	D'	X <sup>2</sup>	p-value	N	r <sup>2</sup>
rs17583889	rs8187710	0.451	4.216	0.04*	248	0.009
rs17583889	rs1883112	0.129	1.970	0.16	236	0.004
rs17583889	rs13058338	0.318	1.101	0.29	247	0.002
rs17583889	rs246221	0.143	1.192	0.27	246	0.002
rs17583889	rs17222723	0.152	0.210	0.65	240	0.000
rs17583889	rs2229774	0.124	7.125	0.008*	248	0.015
rs8187710	rs1883112	0.310	8.066	0.005*	251	0.016
rs8187710	rs13058338	0.180	0.930	0.34	263	0.002
rs8187710	rs246221	0.160	4.746	0.03*	263	0.009
rs8187710	rs17222723	0.954	212.547	0	257	0.414
rs8187710	rs2229774	0.0180	0.007	0.93	265	0.000
rs1883112	rs13058338	0.106	1.870	0.17	257	0.004
rs1883112	rs246221	0.121	3.574	0.05*	255	0.007
rs1883112	rs17222723	0.151	0.848	0.36	245	0.002
rs1883112	rs2229774	0.176	3.740	0.05*	257	0.007
rs13058338	rs246221	0.004	0.002	0.97	267	0.000
rs13058338	rs17222723	0.026	0.307	0.58	257	0.001
rs13058338	rs2229774	0.040	0.620	0.43	269	0.001
rs246221	rs17222723	0.225	4.170	0.04*	259	0.008
rs246221	rs2229774	0.116	0.959	0.33	269	0.002
rs17222723	rs2229774	0.011	0.052	0.82	259	0.000

All seven SNPs were utilised to generate both an LD plot and haplotype analysis (Fig.40). Figure 40 shows that cumulatively all the SNPs are not in strong LD and not inherited as a single haplotype.



**Figure 40: LD plot (A) and Haplotype analysis (B) generated by the SHEsis program<sup>132</sup> using all seven SNPs**

#### 4.7.2 Association testing

Patient status was significantly associated with *RARG* rs2229774 ( $p=0.049$ , Chi-square test;  $p=0.05$ , Fisher's exact test). No association was found between patient status and other variants - *ABCC1* rs246221 ( $p=0.56$ ), *ABCC2* rs17222723 ( $p=0.95$ ), *ABCC2* rs8187710 ( $p=0.44$ ), *HNMT* rs17583889 ( $p=0.18$ ), *NCF4* rs1883112 ( $p=0.28$ ) and *RAC2* rs13058338 ( $p=0.53$ ).

The more accurate logistic regression was used to determine whether any of the variants predicted clinical status. As previously mentioned, logistic regression was found to be better suited to our needs due to its ability to model the determinants of and predict the likelihood of an outcome compared to descriptive correlation for which chi-square tests are most apt. None of the SNPs showed any significant association with patient clinical status (Table 37).

Severity score was significantly associated with *ABCC2* rs8187710 ( $p=0.004$ , Chi-square test) and *ABCC2* rs17222723 ( $p=0.006$ , Chi-square test). However no association was found between patient severity score and other variants - *ABCC1* rs246221 ( $p=0.21$ ), *HNMT* rs17583889 ( $p=0.72$ ), *NCF4* rs1883112 ( $p=0.07$ ) *RAC2* rs13058338 ( $p=0.89$ ) and *RARG* rs2229774 ( $p=0.66$ ).

Logistic regression models were computed firstly for genotype related to the outcome of status and then genotype together with total dose related to the outcome of clinical status.

**Table 37: Logistic Regression Co-efficients used to determine Odds Ratios using Genotype to Predict Outcome**

Covariates	Test Statistic	Odds Ratio <sup>^</sup>	p-value
<i>HNMT</i> rs17583889 C/A	-0.014	2.19e <sup>-07+</sup>	0.989
<i>HNMT</i> rs17583889 C/C	-0.015	1.17e <sup>-07+</sup>	0.988
<i>ABCC2</i> rs8187710 G/A	1.055	1.98 (0.49-6.65)	0.291
<i>ABCC2</i> rs8187710 G/G	1.261	2.20 (0.57-7.05)	0.207
<i>NCF4</i> rs1883112 G/A	0.671	1.46 (0.46-4.35)	0.502
<i>NCF4</i> rs1883112 G/G	-0.572	0.75 (0.26-1.9)	0.568
<i>RAC2</i> rs13058338 A/T	-1.105	0.65 (0.31-1.43)	0.269
<i>RAC2</i> rs13058338 T/T	-0.286	0.73 (0.11-14.23)	0.775
<i>ABCC1</i> rs246221 A/G	-1.037	0.64 (0.26-1.45)	0.300
<i>ABCC1</i> rs246221 G/G	-0.368	0.83 (0.31-2.26)	0.713
<i>ABCC2</i> rs17222723 A/T	-0.235	0.90 (0.40-2.25)	0.814
<i>ABCC2</i> rs17222723 T/T	-0.217	0.78 (0.12-15.34)	0.828
<i>RARG</i> rs2229774 G/A	-0.012	1.5e <sup>-06+</sup>	0.990
<i>RARG</i> rs2229774 G/G	-0.014	3.2e <sup>-07+</sup>	0.989

<sup>^</sup>odds ratios with 95% CI; +sample size too small to compute 95% CI

Odds ratios indicate that despite the genotype, total dose had a statistically significant contribution to the outcome of clinical status (Table 38).

**Table 38: Logistic Regression Co-efficients used to determine Odds Ratios using Genotype and Dose Covariates**

Covariates	Test Statistic	Odds Ratio <sup>^</sup>	p-value
<i>HNMT</i> rs17583889 and Total Dose	2.430	1.002 (1.0006-1.004)	0.015*
<i>ABCC2</i> rs8187710 and Total Dose	2.553	1.002 (1.0005-1.004)	0.011*
<i>NCF4</i> rs1883112 and Total Dose	2.163	1.002 (1.0003-1.004)	0.031*
<i>RAC2</i> rs13058338 and Total Dose	2.363	1.002 (1.0004-1.004)	0.018*
<i>ABCC1</i> rs246221 and Total Dose	2.355	1.002 (1.0004-1.004)	0.019*
<i>ABCC2</i> rs17222723 and Total Dose	2.433	1.002 (1.0004-1.004)	0.015*
<i>RARG</i> rs2229774 and Total Dose	2.247	1.002 (1.0003-1.004)	0.025*

\*statistically significant; <sup>^</sup>odds ratios with 95% CI

The genetic models of variants was examined in the context of their allelic frequency and contribution to outcome (affected cardiac status due to ACT) in Table 39.

**Table 39: Overview of genetic variants, their genetic model and contribution to phenotype**

Gene Variants	Minor Allele Frequency		p-value <sup>#</sup>	Covariates	Odds Ratio	p-value <sup>^</sup>	Type of Genetic Model
	Lit	Study					
HNMT rs17583889	0.13	0.12	0.7682	HNMT rs17583889 C/A	2.19e <sup>-07</sup>	0.989	Multifactorial/ Polygenic
				HNMT rs17583889 C/C	1.17e <sup>-07</sup>	0.988	
ABCC2 rs8187710	0.07	0.24	<0.0001*	ABCC2 rs8187710 G/A	1.98	0.291	Multifactorial/ Polygenic
				ABCC2 rs8187710 G/G	2.20	0.207	
NCF4 rs1883112	0.41	0.35	0.0106*	NCF4 rs1883112 G/A	1.46	0.502	Multifactorial/ Polygenic
				NCF4 rs1883112 G/G	0.75	0.568	
RAC2 rs13058338	0.16	0.14	0.5561	RAC2 rs13058338 A/T	0.65	0.269	Multifactorial/ Polygenic
				RAC2 rs13058338 T/T	0.73	0.775	
ABCC1 rs246221	0.42	0.48	0.0737	ABCC1 rs246221 A/G	0.64	0.300	Multifactorial/ Polygenic
				ABCC1 rs246221 G/G	0.83	0.713	
ABCC2 rs17222723	0.04	0.12	<0.0001*	ABCC2 rs17222723 A/T	0.90	0.814	Multifactorial/ Polygenic
				ABCC2 rs17222723 T/T	0.78	0.828	
RARG rs2229774	0.09	0.11	0.4798	RARG rs2229774 G/A	1.5e <sup>-06</sup>	0.990	Multifactorial/ Polygenic
				RARG rs2229774 G/G	3.2e <sup>-07</sup>	0.989	

## Chapter 5: Discussion

### 5.1 Summary of Main Findings

“Anthracycline-induced cardiotoxicity, while dose-dependent, may be attributed to inherent genetic differences in treated patients” was the thesis statement specified at the beginning of the study. This study sought to discover differences between treated patients to allow for the minimisation of cardiotoxicity due to anthracycline-based chemotherapy.

The risk of ACT has been estimated, according to GLOBOCAN, at 10-26%<sup>15</sup>. The retrospective (79/402=19.7%) and prospective cohorts (38/272=14%) in the present study showed that approximately 17.35% of patients here in the Western Cape were likely affected with ACT. This incidence of ACT may be an underestimation since some patients with initial subclinical damage may later progress to irreversible cardiac damage and subsequent symptoms<sup>17</sup>, and perhaps not be related back to their chemotherapy. The extent to which different population groups were affected with ACT seemed distinguishable – IAs had the highest overall incidence of ACT (20% and 19%), MA patients had a relatively lower incidence (19.8% and 13%) and CA patients had the lowest overall incidence of ACT (16.7% and 10%), in both the retrospective and prospective cohorts, respectively. Therefore, patient risk with regard to clinical co-morbidities were investigated.

Clinical co-morbidities such as hypertension, diabetes, cardiac disease and smoking have previously been associated with increased risk of developing ACT<sup>10,24,48</sup>. In the retrospective and prospective cohorts, the incidence of hypertension was the highest (40% and 43%, respectively) with pre-existing cardiac disease (5% and 7%, respectively) having the lowest incidence. These clinical co-morbidities were stratified into population group in both the retrospective and prospective cohorts; MA patients had the highest overall risk of developing ACT due to clinical co-morbidities with IA patients having a fairly low to low risk. Therefore, these risk factors were not predictive of the outcome of ACT.

Previously, the type of anthracycline-based chemotherapy and dose were reported to be associated with ACT and therefore investigated in the Cape Town cohort<sup>24,25,46,51</sup>. Patients in both cohorts were analysed for type and dose of anthracycline and it was found that in the retrospective cohort, CA patients were most likely to be prescribed doxorubicin and at higher median doses compared to their MA counterparts whereas in the prospective cohort, both MA and IA patients were prescribed doxorubicin at

higher median doses compared to their CA counterparts. Logistic regression showed that both type and dose of anthracyclines were significantly associated with ACT for both the retrospective and prospective cohorts.

The motivation for the investigation of genetic variants associated with susceptibility/resistance to ACT was for a range of reasons which included: that ACT was evident at different incidences in the three local populations of cancer patients receiving anthracycline-based chemotherapy; (ii) previous literature indicating pharmacogenetic association with ACT ; and (iii) evidence of IA women being more susceptible to cardiotoxicity due to ethnically variant polymorphisms pertinent to anthracycline metabolism <sup>6-8,139,140</sup>.

Prospectively recruited patients (n=272) were genotyped for seven SNPs: *ABCC1* rs246221; *ABCC2* rs17222723; *ABCC2* rs8187710; *HNMT* rs17583889; *NCF4* rs1883112; *RAC2* rs13058338 and *RARG* rs2229774. A simple illustration of genetic diversity between the three local populations was evident from statistically significant differences of the MAFs for these markers when compared to literature – while the local CA patients showed no significant differences, the MA patients had 2 SNPs (*ABCC2* rs17222723 and *ABCC2* rs8187710) and the IA patients had 5 SNPs (*ABCC1* rs246221, *ABCC2* rs17222723, *ABCC2* rs8187710, *NCF4* rs1883112 and *RAC2* rs13058338) with significantly different allele frequencies compared to that reported (generally for CA) in the literature. Furthermore, SNPs interrogated in the patient population had shown previous association to ACT however the only SNP that showed an association with patient clinical ACT status was *RARG* rs2229774, which corroborated an earlier Canadian GWAS investigating ACT in childhood cancer <sup>7</sup>. This significance however was lost when the more robust logistic regression to predict outcome was applied.

## 5.2 Retrospective Cohort Analysis

Data analysis was performed on the entire cohort (n=402) and stratified into the local population groups. The recruitment sites, two government hospitals in Cape Town, South Africa, were attended by predominantly individuals of MA and to a lesser degree, IAs and CA individuals. MA refers to an admixed population of origins as diverse as European (including Dutch, French, German and English), Asian and indigenous Khoisan, whereas IA refers to a population group of native or indigenous Africans with various ethnicities such as Zulu and Xhosa, and CA refers to a population group of European origin, mentioned earlier <sup>141</sup>. Not only does population stratification improve accuracy for larger scale association studies, but it may also aid

in delineating extreme phenotypes due to the unique genotypes of the constituent local populations <sup>6,142</sup>.

There were only seven males in this cohort – this was due to the fact that they are only rarely affected with breast cancer, the prevalence is reported to be approximately 1% in the literature <sup>14,34</sup>.

Additional demographic factors such as age and BSA were analysed. While the overall median age at diagnosis for this cohort was 51 years, which was in agreement with South African Census statistics <sup>143</sup>, this is comparatively younger than the published worldwide statistics of 65 years and older <sup>15</sup>. The IA cohort had a median age of only 44 years. Young age at diagnosis in the IA patient cohort may be attributed to a range of factors which is outside of the scope of this research but may include risk factors such as delayed onset of childbirth and genetic influences where hereditary factors such as *BRCA1/2* mutations (amongst those in other genes) may be a contributing factor <sup>144,145</sup>. Similarly, BSA and by extension, body mass index (BMI), were found to be consistently higher than normal especially in the MA and IA sub-cohorts which may be attributed to two possibilities – i) ethnic differences in body form for local populations that do not correlate with worldwide standards, therefore making a universal cut-off value inapplicable, and/or ii) the possibility of the adoption of an unhealthier lifestyle due to e.g. urbanisation and modernization <sup>146,147</sup>.

Clinical co-morbidities such as hypertension, diabetes, cardiac disease and smoking, shown to increase the risk of developing ACT previously, were also investigated. Logistic regression models based on our retrospective data did not substantiate these findings <sup>24,26,36,148</sup>. Nevertheless, both MA and IA patients had higher rates of these co-morbidities compared to their CA counterparts which may indicate shared risk factors (including genetic) in these two groups (i.e. MA and IA) resulting in greater susceptibility to certain lifestyle-induced diseases, or perhaps due to inadequate access to healthcare due to socioeconomic circumstances.

Risk factors such as type and dose of anthracycline were also assessed. The anthracyclines, doxorubicin and epirubicin, have different safety profiles. Due to a shorter terminal half-life and more rapid total body clearance <sup>22,26,30</sup>, epirubicin is considered “safer” and given at increased doses. However, our analysis shows that neither of these agents are entirely safe – 138 patients were treated with doxorubicin and 37 were affected (27%) compared to 264 patients treated with epirubicin, 42 of whom were affected (16%). This relative (minimal) improvement (i.e. 16% versus 27%) in ACT-affectation status warrants the investigation of alternatives such as

liposomal-encapsulated anthracyclines or the parallel provision of a cardio-protectant  
75,76,98.

Patients' clinical status in terms of ACT was either 'affected' or 'unaffected' based on numerous factors which included LVEF decline. It emerged that IAs at 20% may be marginally more susceptible compared to MA at 19.8%, with Caucasians at 16.7% being the relatively least susceptible. Previous evidence of ethnically variant polymorphisms that influence doxorubicin pharmacokinetics and pharmacodynamics suggest that population-based differences may be expected<sup>6,139,140</sup>.

Although relied on heavily in this study, LVEF, as a measure of cardiac function may prove to be unreliable, whereby 56/402 patients (14%) had significant decreases in LVEF ( $\geq 10\%$ ) yet only seven out of the 56 patients exhibited clinical signs and symptoms of cardiac dysfunction. Conversely, 79/402 patients (20%) were deemed to be clinically "affected" post-treatment (i.e. exhibiting signs of cardiac dysfunction/impairment) – however, only 56 out of the 79 patients had significant LVEF decline – a failure rate of 29%, indicating that LVEF may be ineffective as both an early and sensitive measure of cardiac dysfunction, specifically ACT. Despite LVEF being the widely accepted measure of cardiac function, its lack of sensitivity and inherent variability has been mentioned previously<sup>149,150</sup> and other alternatives such as the measurement of global longitudinal strain as a more sensitive and reproducible prognostic indicator of ACT has been suggested<sup>69,151</sup>.

Therefore, in this study, the "unaffected" status of patients was not deemed definitive without an echocardiography to provide confirmation. Some measure of association could be lost due to the potential inaccuracy in phenotyping. In trying to circumvent this issue and provide clarity, the severity score or classifier was developed based on previous literature (CTCAE grading) and the clinical phenotype of the cohort<sup>51-54,152</sup>.

Logistic regression models developed using retrospective patient data pointed to the following factors contributing to the final phenotype: hypertension, smoking, type of chemotherapy (i.e. total dose of anthracyclines: either doxorubicin, or epirubicin), and the change in LVEF from baseline to after chemotherapeutic administration, which have all been previously indicated<sup>24,51,87,148</sup>. However, in a multivariable model, the only factor that showed robustness was the LVEF decline – nevertheless this still does not indicate its utility as an early indicator of cardiotoxicity as LVEF decline was used for determining clinical status and the severity score of patients.

ANOVA type II tests showed that pre-chemo LVEF combined with population group contributed to the post-chemo LVEF measure which may indicate that different LVEF

ranges for different population groups may be more accurate in determining cardiac clinical status. Furthermore, when the pre-chemo LVEF was combined with severity score, population group and type of first-line chemotherapy, a fairly robust model of prediction of post-chemo LVEF emerged. This may have utility in financially-constrained hospital settings where only one measure of LVEF (pre-chemo) is possible.

### **5.3 Prospective Cohort Analysis**

Anthracycline-naïve patients (n=272) were prospectively recruited from both GSH and TBH Hospitals in the Western Cape Province of South Africa.

Similar to the retrospective cohort analysis, patient clinical histories were extensively analysed. Infiltrating or invasive ductal carcinoma was found to be most frequently diagnosed in both cohorts (71% of the entire cohort), in accordance with worldwide statistics<sup>153</sup>. The median age of diagnosis in this cohort was 50 years with the IA cohort again being significantly younger (44 years) compared to both the MA (51 years) and CA (59 years) patients. The BSA of this cohort was also higher and in this instance, to a greater degree for IA (1.81m<sup>2</sup>) and CA (1.84m<sup>2</sup>) compared to their MA counterparts (1.74m<sup>2</sup>) (See Table 30). Age at diagnosis and BSA are similar to the trends observed in the retrospective patient cohort.

While the retrospective cohort showed a high number of patients with unknown HIV status, the prospective cohort showed a higher proportion of IA women (43%) with HIV. This may reveal easier accessibility and increased testing which is useful for patient care and management. However, the differential between HIV positivity in the cohort (43%) and the general reported incidence of 18% in this province is worthy of note<sup>154</sup>.

The co-morbidities present in this cohort, which include hypertension, diabetes mellitus, cardiac disease and smoking status provided interesting insights when they were analysed within the population groups. IA patients were, on average, younger, but they were also found to be at the lowest risk for hypertension, diabetes and smoking. However, baseline cardiac incidental findings were highest in this group (13%). These cardiac incidental findings could be attributed to poor previous clinical care and subsequent subclinical cardiac dysfunction and/or the increased genetic likelihood of cardiac insufficiency<sup>155,156</sup>.

Incidental cardiac findings were both of interest and a confounding factor that could not be disregarded as they had a bearing on the outcome phenotype (cardiac status).

These findings ranged from the more serious left ventricular hypertrophy where ACT would be potentially greatest, to the lesser but still serious rhythm abnormality. Despite this, LVEF for all these patients were 'normal' allowing for treatment to be initiated – only one patient had an inconclusive LVEF measure and this was presumably in response to rhythm abnormality.

The patient standard of care at the beginning of the study included LVEF measurement at both baseline (before chemotherapy) and again after three or four cycles of chemotherapy. However, protocol changes due to change in management and an overburdened healthcare system resulted in only one LVEF measurement (baseline) recorded for a number of the prospective cohort (68%). Furthermore, patient overload on the Department of Nuclear Medicine at TBH and GSH resulted in some patients without any LVEF measure. Chemotherapy was administered regardless of cardiac status (either before or after treatment) as the treatment was deemed to be of greater need i.e. to treat cancer compared to the potential danger of cardiotoxicity. This, of note, is the standard of care in most public hospitals in the country.

However, the number of cardiac incidental findings in seemingly healthy patients (Table 32) at baseline together with significant rates of post-chemotherapy affected (ACT) patients (exhibiting signs of cardiotoxicity) emphasises the need for instituting effective measures of cardiac function and, specifically, repeated measures of LVEF.

The model of prediction of post-chemotherapy LVEF was developed using retrospective patient data – notably the following variables: pre-chemotherapy LVEF, severity score, population group and type of first-line chemotherapy. This statistical model was validated in prospective patients (n=87) with no missing variables and allowed for a fairly utilizable estimation of post-chemotherapy LVEF with an accuracy of 82%. The prediction of post-chemotherapy LVEF could allow for the change in LVEF and subsequent clinical cardiac status (i.e. dysfunction/impairment) to be ascertained. This predictive model could be utilised in patients with missing secondary LVEF measures (post-chemotherapy LVEF) for improved patient outcomes and may ultimately warrant further development.

There were 14% affected individuals in this cohort. However this may be an underestimation due to the likelihood of subclinical damage which can manifest later and both LVEF measure and clinical symptoms may not be indicative at the stage that patients were being assessed <sup>56,58,106</sup>. Nevertheless, IAs may have an increased risk of ACT as they were proportionally most affected (19%) compared to MA (13%) and

CA (10%), which is also in agreement with the retrospective cohort findings and the literature <sup>6,140</sup>.

After patient history, and patient status regarding ACT, genotyping of patient DNA was undertaken for an association study. Firstly, SNP MAFs for each population group were compared to MAFs (Reference population: ALL) derived from both dbSNP and Ensembl <sup>81</sup>. The allelic frequencies of five SNPs (*ABCC1* rs246221, *ABCC2* rs17222723, *ABCC2* rs8187710, *NCF4* rs1883112 and *RAC2* rs13058338) in IAs and two SNPs (*ABCC2* rs17222723 and *ABCC2* rs8187710) in MA patients were significantly different compared to literature and Ensembl <sup>81</sup> reiterating the need for the inclusion of African and/or local populations in worldwide databases <sup>157</sup>. The MAFs of the investigated SNPs for the CA patients in this study were in accordance with the MAFs reported in both Ensembl and dbSNP (Population: ALL). Secondly, Hardy-Weinberg Equilibrium calculations were conducted and two SNPs – *HNMT* rs17583889 and *NCF4* rs1880112 were found not to be in Hardy-Weinberg Equilibrium in the cohort. While genotyping error is an improbable reason for these deviations (as confirmed by Sanger sequencing), the relatively small cohort size may explain this deviation.

The variant, *RARG* rs2229774 was found to have a strong association with anthracycline-induced cardiotoxicity in a genome-wide association study conducted by Aminkeng *et al.* <sup>7</sup> on childhood cancer patients of both European and non-European (including Asian, African-American and Hispanic) ancestry assessing cardiac function using the CTCAE grading and ECHO measures. The present study also found a statistically significant association of *RARG* rs2229774 with clinical status pertaining to ACT (Chi-square test, p=0.049). However, the more accurate logistic regression models found no association with this variant or any of the other variants and ACT status.

*ABCC1* rs246221 was not associated with clinical status pertaining to ACT in this study, however a previous study by Semsei *et al.* <sup>158</sup> found this variant to be associated with anthracycline-induced left ventricular dysfunction in Hungarian paediatric patients with acute lymphoblastic leukaemia. Aside from a different population and the use of ECHOs to show diminished cardiac function, patients were followed for a median of 6.3 years. Therefore the lack of association of this variant with ACT in our study may indicate that it would be worth following up on our patients longer term, and assessing for ACT (our patients were only followed for 1-3 years). Similarly, Vulsteke *et al.* <sup>159</sup> found *ABCC1* rs246221 to be associated with an LVEF

decline of >10% in a European CA cohort of breast cancer patients treated with epirubicin and followed for a median of 3.62 years. While our study cohort had breast cancer patients, treatment with epirubicin and the outcome of LVEF decline >10%, our cohort consisted of mostly African-derived populations.

Wojnowski *et al.*<sup>8</sup> found the variants *NCF4* rs1883112, *RAC2* rs13058338 and *ABCC2* rs8187710 to be associated with ACT in a German patient population treated with anthracyclines for non-Hodgkin lymphoma (NHL), and followed up for a median of more than three years, where arrhythmias and cardiac failure were classified as acute and chronic ACT, respectively. Again the divergent European-based population and longer follow up time may explain the lack of association with these variants and ACT in the present study. Similarly, Leong *et al.*<sup>105</sup> found an increased risk of ACT associated with *RAC2* rs13058338 and *ABCC2* rs8187710.

The variant, *ABCC2* rs17222723, had been previously associated with acute cardiotoxicity<sup>83,137,160</sup> due to its role in drug transportation of cytotoxic agents into and out of the cell. However, the present study failed to find an association between this SNP and cardiotoxicity despite its sensitivity to acute ACT.

Similarly, the variant *HNMT* rs17583889, was found to have an association with ACT in Canadian paediatric cancer patients where cardiac function was assessed using both CTCAE guidelines and ECHOs<sup>10</sup>. However, in the current study of adult women, this variant showed no association with ACT and this may be explained by not only ancestry of the population compared to our cohort but also that paediatric cases may have greater susceptibility to cardiac damage due to the developing heart. It has been shown that paediatric hearts have inadequate compensatory mechanisms for cardiomyocyte loss allowing cardiac damage from anthracyclines to be potentially progressive thereby resulting in significant morbidity and/or mortality<sup>161</sup>.

Furthermore as suggested in the Aminkeng *et al.*<sup>6</sup> article, genetic differences in key genes in African populations may account for increased sensitivity to both ACT and CHF. Differences in MAF as shown in the study between IA patients and literature illustrates these genetic dissimilarities. Beyond the variants discussed in this study, Aminkeng *et al.*<sup>6</sup> showed that CYP enzymes', involved in the metabolism of 40-60% of all drugs, varied expression due to genetic differences may contribute to predisposition to several ADRs and poor response rates in African compared to European populations. The pharmacogenomic heterogeneity of African populations across different ethnic groups and geographical regions may contribute to differences in drug efficacy and safety profiles.

Variables that appeared to increase the odds of the outcome (patient clinical status) included significant LVEF decline (>10%) and total anthracycline dose ( $p=9.9e^{-05}$  and  $p=0.0164$ , respectively). The logistic regression of a multivariable model with both these factors was found to have an odds ratio of 1.704 and a p-value of 0.000164 thereby indicating a strong association of LVEF change and total anthracycline dose to level of cardiotoxicity (i.e. patient clinical status related to cardiotoxicity). Despite the inadequacy of LVEF as a sensitive measure of ACT, it has some utility in predicting the outcome of cardiotoxicity. Similarly, the dose of anthracycline, also predicts the outcome of cardiotoxicity – a finding that has been supported previously<sup>24,51,53</sup>.

## 5.4 Study Limitations

General limitations of the study were:

- i) relatively small sample size – despite the recruitment of 272 prospective patients, their population stratification may have decreased statistical power of certain associations;
- ii) short median follow-up – the study in fulfilment of a PhD with a finite completion period included the recruitment of prospective patients before anthracycline-based treatment as well as follow-up after treatment which may have been too short a timeframe to assess progressive late-onset ACT;
- iii) the lack of information pertaining to lifestyle factors such as stress and exercise which may have had a bearing on cardiac function and/or susceptibility to cardiotoxicity. For example, studies indicate that aerobic exercise may mitigate cardiovascular dysfunction<sup>162,163</sup>;
- iv) lack of two LVEF measures for every patient – despite the insensitivity of the LVEF measure in some instances, the lack of a second measure complicated patient phenotyping with regard to ACT;
- v) attributing differences in genotypes to self-reported ethnicity – which may decrease population-specific significance. Ancestry Informative markers may have been useful in delineating the different population groups rather than the reliance on self-reporting<sup>164</sup>. The limitation of attributing differences in genotypes to self-reported ethnicity is that SNPs with population-specific significance may be lost or diluted in the sub-cohorts (stratified according to population group). Furthermore, patient follow-up at a later stage could reveal late-onset ACT and therefore an affected case which may

contribute to non-associated SNPs finding statistical significance, particularly within the heterogeneous local population groups.

## **5.5 Recommendations**

Despite the study's focus on the development of ACT, other issues related to patient health emerged. The lack of HIV status in a significant fraction of the retrospective cohort, is a concern given that South Africa has one of the largest epidemics in the world <sup>154</sup>. As was noted in the prospective cohort – up to 43% of women in the IA sub-cohort were HIV positive. Although not focused on in this thesis – HIV status or perhaps the status of an individual's immune system (e.g. AIDs versus non-AIDs in HIV positive subset) may contribute to ACT-susceptibility, and makes for a reasonable follow-up project with public health relevance.

In terms of managing ACT, patients with suspected or potential cardiotoxicity may be at a critical point whereby reversible cardiac damage may progress to irreversible, chronic or sometimes life-threatening cardiomyopathy, yet few patients were referred to cardiology clinics for follow-up and confirmatory ECHOs <sup>25,60</sup>. The inherent variability and lack of sensitivity of LVEF as a measure of cardiac function warrants the consideration of alternatives such as ECHOs or tissue-doppler imaging as a truer measure of cardiotoxicity. We therefore recommend routine ECHOs before and after chemotherapy as standard of care in the government setting for improved patient outcomes and decreased chronic cardiac care costs.

## **5.6 Future Directions**

While this study provided valuable insights into the genetic heterogeneity of our African populations and their increased risk of developing ACT, this is merely a good starting point for future work.

Confirmed affected and non-affected (normal) patients' DNA may be utilised for GWAS to determine the nuances of their susceptibility to ACT. Patients will be stratified according to population group, confirmed by ancestry informative markers and their GWAS data analysed.

Additional patients may be recruited with the aim of improving cardiac assessment – ECHOs before and after treatment as well as submitting patient samples to a panel of biomarkers. These patients cardiac status can then be correlated to genotyping for the SNPs discussed in this study. Furthermore, the model developed in this study may be validated using actual post-chemotherapy cardiac measures.

While a genotypic change provides information that may be pertinent to susceptibility or protection, the level of expression may confer to what extent this is the case. Quantitative PCR may provide valuable insight/s into expression of genes associated with ACT.

## Chapter 6: Supplementary Study – Testing of Biomarkers as a Measure of Cardiac Injury

### 6.1 Introduction:

Cardiac biomarkers found in blood had been initially used to detect cardiotoxicity in animal models and in pilot clinical studies, however they have the potential to be a more accurate measure of early cardiotoxicity in the routine management of patients<sup>17,18,22,37,69</sup>. The advantages of biomarker assays is that they are standardized thereby minimizing technical variability to some extent, and they are also minimally invasive and easily repeated. Serial monitoring of serum biomarker concentrations have been proposed as a more specific and sensitive modality of cardiotoxicity detection<sup>24,69</sup>, perhaps in place of LVEF (and the means of currently measuring this parameter), as discussed earlier in the thesis. Biomarkers that are currently being utilised in the assessment and detection of cardiotoxicity include: natriuretic peptides, troponins, and the ST2 cardiac biomarker<sup>161,165-167</sup>, which are reviewed hereunder

#### 6.1.1 Brain Type Natriuretic Peptide (BNP)

Natriuretic peptides play an important regulatory role in cardiovascular (Fig. 41) and renal homeostasis, as well as fatty acid metabolism and subsequent maintenance of body weight<sup>168</sup>. Natriuretic peptides belong to a group of structurally similar yet genetically heterogeneous peptide hormones, namely: atrial, brain and C-type (ANP, BNP, CNP respectively)<sup>69,168</sup>.

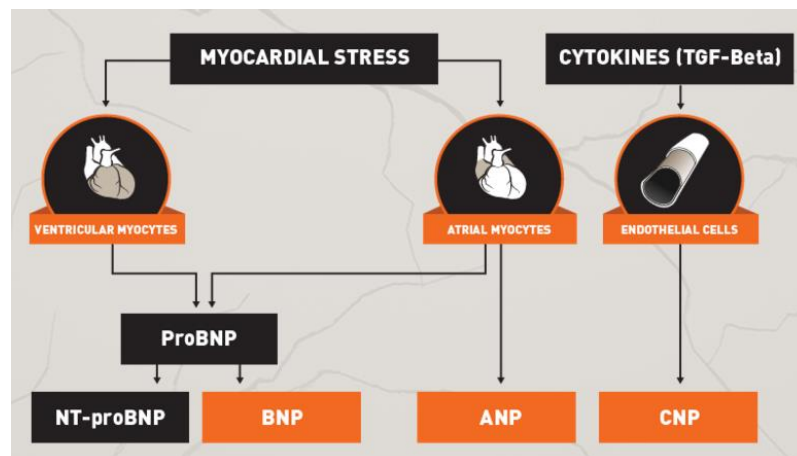


Figure 41: Natriuretic peptides involved in Cardiac Failure<sup>169</sup>

All three peptides (Fig. 42) contain a 17 amino acid disulphide ring with variable C and T terminal regions<sup>170</sup>.



genes, involved in NT-proBNP processing, have been suggested as plausible explanations for lowered NT-proBNP levels in African Americans, where these functional variants exist at a higher frequency <sup>164</sup>.

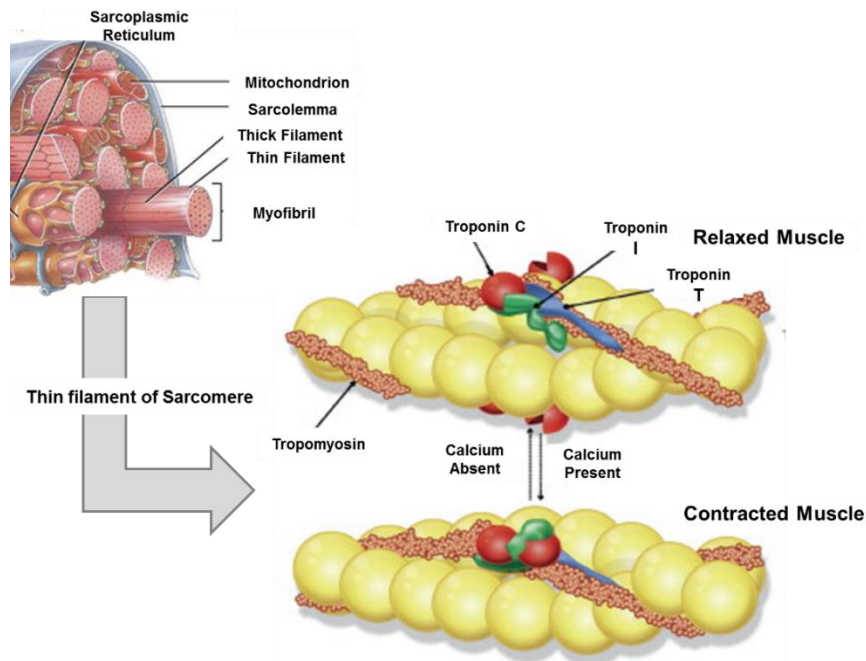
During the first three months of chemotherapy, increased levels of BNP were reported to be associated with abnormal LV thickness-to-dimension ratio which may indicate LV remodelling; in this regard, studies have shown abnormal LV structure years after chemotherapy <sup>24,136</sup>.

However Plana *et al.* <sup>69</sup> found that the association between BNP levels and LV function were prone to inconsistencies.

### **6.1.2 Troponin T**

Cardiomyocyte damage results in the increase of circulating cardiac troponin in the blood allowing for plasma and serum to be utilized for biomarker testing to assess cardiac health <sup>136</sup>. Cardiac troponin T-levels are generally undetectable in healthy individuals but are elevated where there is myocardial injury, possibly indicating irreversible cardiomyocyte necrosis <sup>24,39,136</sup>. Negative troponin concentrations have been shown to be indicative of low risk of cardiomyopathy – pointing to the sensitivity of troponin T as a measure of even early or subclinical cardiac damage which may allow for treatment while damage is still reversible <sup>37,136,176</sup>.

Troponins are proteins found in both cardiac and skeletal muscle. The troponin complex consists of thin filaments in striated muscle which is complexed to actin. There exist three types of troponins: Troponin T which is tropomyosin binding, Troponin I which inhibits binding between actin and myosin, and Troponin C which is calcium binding allowing for muscle contraction <sup>177</sup>.



**Figure 43: Visualization of cardiac muscle and the different Troponin molecules, adapted from Shave *et al.* <sup>177</sup>**

Despite females reportedly having a greater risk of developing ACT, males are more likely to have increased cardiac troponin levels due to anthracyclines indicating that together with the difference in LV mass between the sexes, an attempt should be made to devise population and gender based Troponin T values correlated with cardiac assessment/function <sup>136</sup>. Furthermore, in addition to cardiac troponin levels being significantly elevated in males, especially those with irradiation near the heart, this biomarker was raised in correlation with higher cumulative doses of anthracyclines <sup>136</sup>.

Cheung *et al.* <sup>136</sup> were not able to elucidate if troponin release was due to anthracycline-induced apoptosis of cardiomyocytes or due to consistent myocardial injury causing troponin release into the bloodstream.

Nevertheless, genotyping revealed that the *CYBA* rs4693 CT/TT genotype was significantly associated with increased cardiac troponin levels compared to the CC genotype, whereas the variants in *RAC2* (rs13058338) and *NCF4* (rs1883112) had no association with troponin levels <sup>136</sup>. The *CYBA* rs4673 CT/TT genotype has been associated with high sensitivity cardiac troponin levels which functionally translate to worse left ventricular longitudinal strain <sup>136</sup>.

### **6.1.3 Suppression of Tumorigenicity 2 (ST2)**

The “Suppression of Tumorigenicity 2” or ST2 marker is an interleukin-33 receptor, part of the interleukin-1 receptor family, and is a cytokine secreted in response to cellular damage, particularly cardiovascular stress and fibrosis <sup>166</sup>. While it has a cardiovascular role, ST2 is also involved in inflammatory and immune processes <sup>166</sup>.

While severe cardiotoxicity during anthracycline-based therapy may result in its early discontinuation, cardiotoxicity may also present years after treatment <sup>24,41</sup>. Cardiac troponin T as a measure of acute cardiotoxicity, may benefit from an additional, sensitive biomarker such as ST2; the latter of which is a powerful predictor for death due to CF, and may be especially useful to detect late onset ACT <sup>41,166</sup>.

While careful monitoring of cardiac function, utilisation of a specific type of anthracycline and limiting cumulative anthracycline dose may mitigate risk of developing ACT, it is likely that cardiotoxicity may still occur regardless of these efforts. Therefore, other measures need to be utilized for the reduction or prevention of cardiotoxicity at the clinical level.

This aspect of the study was to determine whether biomarkers, in this instance NT-proBNP or BNP may serve as a more accurate measure of cardiac injury and cardiotoxicity.

## **6.2 Materials and Methods:**

### **6.2.1 The Study Cohort:**

Forty-two patients from the prospective cohort with adequate biological specimen before and after chemotherapy were selected for the biomarker study.

Peripheral whole blood (3ml) was collected in Greiner Bio-One Vacuette Venous Blood Tubes (Lavender K<sub>3</sub>EDTA) from patients which enable an intact and stable specimen to be collected for plasma for biomarker testing.

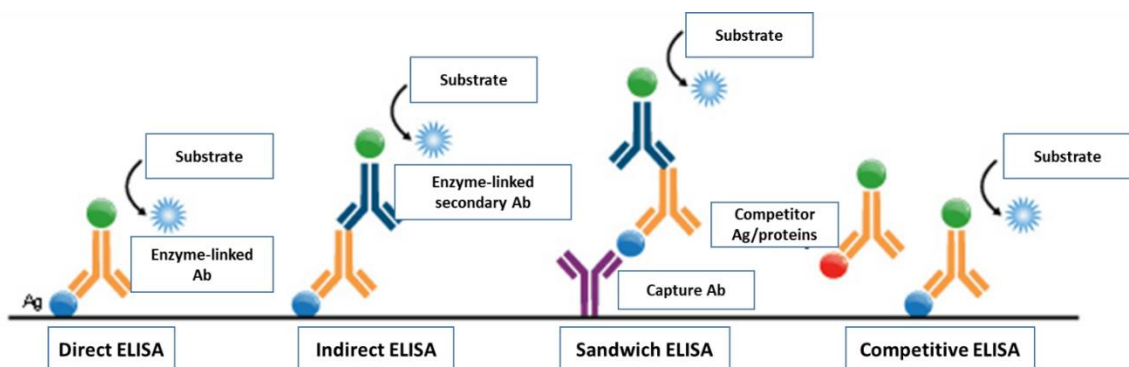
### **6.2.2 Determination of cardiac biomarker of injury levels using peripheral blood**

The Biomedica® Competitive Enzyme Immunoassay (Biomedica Immunoassays, Austria) was used to detect level of NT-proBNPs in order to determine the extent of cardiac injury.

### 6.2.2.1 Principle of ELISA

The method of Enzyme-Linked Immunosorbent Assays (ELISA) or Enzyme Immunoassays (EIA) dates to the 1960s and utilises the combined specificity and sensitivity of antibodies and/or antigens and enzymes allowing for inferences of either antigen or antibody concentration to be made <sup>178-180</sup>. ELISAs can be either used to detect the presence of antigens specifically recognized by an antibody or vice-versa. A generalized ELISA consists of the following: Microtiter plates are first coated with antigen, unbound sites are then blocked to prevent non-specific binding, primary antibodies are added to the wells and finally a secondary antibody which is conjugated to an enzyme, is added <sup>179</sup>. The enzyme acts as the reporter label <sup>178</sup>. This conjugate usually produces a colour change when a substrate is added that ultimately indicates a positive result that can be more accurately quantified with an ELISA plate-reader.

There are four types of ELISA that can be used depending on the object of the experiment: Direct ELISA, Indirect ELISA, Competitive ELISA and Sandwich ELISA <sup>179</sup>. Direct ELISAs involve antigen attachment to the microtiter plate and the addition of an enzyme-linked antibody, allowing for a rudimentary measurement of sample concentrations <sup>179</sup>. Similarly, Indirect ELISAs involve antigen attachment to the solid phase, but the addition of an unlabelled primary antibody with the addition of an enzyme-linked secondary antibody allows for greater specificity in antibody detection <sup>179</sup>. Greater specificity is achieved with Competitive ELISAs whereby “competing” antibodies or proteins are added <sup>179</sup>. The last type of ELISA is both sensitive, specific and amenable to high-throughput platforms – Sandwich ELISAs involve a solid phase coated with capture antigen, the addition of samples with known or unknown antigens in a matrix or buffer followed by the addition of an enzyme-conjugated antibody that allows for a quantifiable detection signal <sup>179</sup>.



**Figure 44: Schematic illustration of the generalized principles of the four types of ELISAs (Image adapted from <sup>181</sup>)**

### **6.2.2.2 Technique of Detection of N-terminal pro B-Type Natriuretic Peptide (NT-proBNP) using a Competitive ELISA**

Peripheral blood was centrifuged at 2500 rpm for 20 minutes at 4°C allowing for separation of plasma. BNP fragments are stable in plasma and serum and therefore centrifugation occurred soon after procurement of sample. Plasma was then stored at -80°C until needed. The plasma may be subjected to five freeze-thaw cycles without compromising sample integrity. The reliability of haemolysed blood samples in determining levels of NT-proBNP is disputed<sup>182</sup>; samples which were suspected of haemolysis were therefore collected and recorded as such, and utilized nevertheless.

The Biomedica assay was performed according to manufacturer's instructions<sup>168</sup>. The wash buffer, standard and control were reconstituted before use. The wash buffer was diluted 1:20 and both the standards (synthetic human BNP fragment at various concentrations (pmol/l) and control (synthetic human BNP fragment) were reconstituted with 200ul of distilled water for 20 min at room temperature, mixed gently and then stored at -25°C. A volume of 150ul of Assay Buffer was added to all wells except the blank in a plate coated with polyclonal anti-BNP fragment antibody followed by 30ul of either samples, control or standards to each well except the blank. This was followed by the addition of 50ul of conjugate (synthetic BNP fragment –HRPO with red dye) to each well except for the blank. The plate was then well covered and incubated overnight at 4°C in darkness. The wells were then aspirated and washed four times with 300ul of diluted wash buffer using an ELISA plate washer (Biotek® Microplate Washer, USA). A volume of 200ul of substrate [Tetramethylbenzidine (TMB) solution] was then added to each well and then incubated for 20 minutes at room temperature in the dark. Subsequently, 50ul of stop solution (Sulphuric acid, 0.18M) was added to each well, shaken well and then placed on the ELISA plate reader capable of determining absorbance at 450nm with a correction wavelength at 630nm. The stop solution can alter the pH therefore inactivating the enzyme and halting the reaction. The correction wavelength allows for non-specific emissions and interference signals to be eliminated thereby giving the correct absorbance of proteins of interest. The assay was performed in duplicate.

#### **6.2.2.3 Statistical Analysis for the determination of NT-proBNP levels**

Results were analysed by measurement of the optical density (OD) of each well. OD values derived from the standards were used to construct the standard curve. Raw data was transformed and analysed using the 4PL algorithm (GraphPad Prism 6) and unknown samples were interpolated using the standard curve. Sample concentrations

were then derived from the antilog of interpolated values. Reference ranges derived from the standards for the assay were specific to the run and could not be utilised across assay runs.

NT-proBNP levels were assessed as transformed log values and as quartiles specific to population as previously done <sup>127</sup>.

### **6.3 Results:**

Incidental cardiac findings and the missing LVEF measures for some patients prompted a pilot study for the assessment of a sub-group of patients at baseline and after chemotherapy, and where blood samples were collected at these two time points, using a cardiac biomarker to determine if there was any cardiac injury in asymptomatic patients.

#### **6.3.1 Determination of cardiac biomarker of injury levels using EDTA-plasma**

Forty two patients were assessed at both baseline and post-chemotherapy for BNP levels using EDTA-plasma from peripheral blood. Raw values from the ELISA were log-transformed and patient concentrations of NT-proBNP were inferred from the fourth-party logistics (4PL) Algorithm using GraphPad Prism 6 <sup>183</sup>. Multivariable linear regression was utilized for the association between population group (predictor) and biomarker levels (outcome). Covariates for the multivariable models included: population group, age, BMI, hypertension, diabetes and smoking status.

#### **6.3.2 NT-pro B-Natriuretic Peptide (NT-proBNP) concentration**

Patients were stratified according to their hypertensive status (Table 40) as hypertension due to ventricular stretching of the myocardium may increase the release of BNPs <sup>175</sup>.

**Table 40: Clinical, demographic and experimental information pertaining to the patients assessed for BNP levels**

Patient ID	Age at Diagnosis	Population Group	Pre-existing CD	Diabetes	Smoking	Other Meds	LVEF Pre	Type of Chemo	Dose	Clinical Symptoms Post	BNP Concentration	
											Pre	Post
<b>Non-Hypertensives</b>												
ACT217	53	MA	N	N	Y	None	57	Epi	690	Severe SOB	571.6	647.8
ACT219	55	MA	N	N	N	None	65	Dox	420	.	363.3	281.1
ACT220	61	MA	N	N	N	None	68	Dox	400	DECEASED	OR	296.1
ACT222	50	MA	N	N	Y	None	65	Dox	480	.	187.2	276.2
ACT227	41	MA	N	N	Y	None	60	Dox	460	.	112.4	188.1
ACT228	50	IA	N	N	N	None	65	Dox	400	.	227.9	562.1
ACT229	54	MA	N	N	N	None	68	Dox	440	.	344.9	55.2
ACT230	58	MA	N	N	N	None	67	Dox	330	.	343.8	267.4
ACT232	69	MA	N	N	N	None	55	Epi	440	Borderline ECG	218.8	415.3
ACT234	68	MA	N	N	N	None	55	Dox	600	.	176.2	288.1
ACT238	52	MA	N	N	N	None	60	Dox	330	.	593.6	402.6
ACT240	30	MA	N	N	N	None	55	Dox	285	Confirmed LVH	500.5	513.3
ACT241	51	MA	N	N	Y	None	67	Dox	600	.	397.9	386.8
ACT245	57	MA	N	N	N	None	60	Dox	270	Borderline ECG	161.0	113.1
ACT246	49	IA	N	N	N	None	60	Dox	330	Potential LVH	662.0	224.3
ACT248	44	IA	N	N	N	None	60	Dox	460	.	206.5	386.8
ACT252	73	MA	N	N	Y	None	53	Epi	330	Confirmed Acute MI	366.3	95.8
ACT259	43	MA	N	N	Y	None	58	Epi	840	.	895.8	440.8
ACT260	63	MA	Y	Y	N	NIDDM meds	55	Dox	345	Chest pain	247.2	151.9
ACT263	43	MA	N	N	N	None	60	Dox	460	.	OR	95.8
ACT264	57	MA	N	N	N	None	55	Epi	375	.	312.7	43.8
ACT272	44	MA	N	N	Y	ARVs	69	Dox	320	.	738.4	540.8
<b>Hypertensives</b>												
ACT215	62	MA	N	N	N	HCTZ, Atenolol, Enalapril, Zocar, Amlidopine	73	Dox	400	.	432.9	48.5
ACT221	63	MA	N	N	N	Hypertensive meds, Simvastatin	77	Dox	420	.	290.0	175.4
ACT223	37	IA	N	N	N	Amlidopine, ARV	55	Epi	520	.	89.5	146.0
ACT224	58	MA	N	Y	N	Hypertensive meds	67	Dox	400	.	146.0	1015.6

**Table 40 continued: Clinical, demographic and experimental information pertaining to the patients assessed for BNP levels**

ACT225	54	MA	N	N	N	Dispirin, Amlidopine	60	Dox	380	.	159.7	217.0
ACT226	50	MA	N	N	Y	Ridaq, Pharmapress	67	Dox	330	Abnormal ECG; Sinus Tachycardia	93.7	465.5
ACT231	44	MA	N	N	Y	None	65	Dox	330	.	387.8	392.3
ACT244	47	MA	N	N	N	Ridaq	55	Epi	540	Abnormal ECG; Prolonged QT	324.2	140.9
ACT249	56	MA	N	Y	Y	Ridaq, Pharmapress, Glibendamide	58	Epi	780	.	362.3	43.1
ACT250	43	IA	N	N	N	Ridaq, Atenolol, Zocar; Simvastatin, Enalapril	none	Dox	440	.	362.3	436.4
ACT251	63	MA	N	N	N	Ridaq, Atenolol	60	Epi	500	Borderline ECG	187.1	129.3
ACT253	51	MA	N	N	Y	TB Drug Regimen 1; Ridaq, Atenolol	71	Dox	380	Potential MI	523.2	715.5
ACT254	48	MA	N	N	N	Ridaq, Atenolol	66	Dox	345	.	234.5	139.0
ACT255	63	MA	N	N	Y	Hypertensive Meds	60	Dox	400	.	276.8	305.2
ACT257	40	MA	N	N	N	Ridaq, Pharmapress, Atenolol	65	Dox	460	.	568.6	203.2
ACT261	67	IA	N	N	N	None	65	Dox	450	.	388.2	289.0
ACT265	38	MA	N	N	N	Pharmapress	65	Dox	400	.	550.5	490.3
ACT266	60	MA	Y	N	N	Atenolol	60	Dox	400	.	345.0	236.8
ACT269	45	MA	N	N	Y	ARVs, Pharmapress	64	Dox	360	.	132.2	496.5
ACT273	43	MA	N	N	N	Ridaq, Pharmapress	50	Dox	400	Potential LVH	1139.8	676.5

\*SOB = shortness of breath; ECG= echocardiogram; LVH= left ventricular hypertrophy; MI= myocardial infarction; NIDDM= non-insulin dependent diabetes mellitus; ARVs= antiretroviral; HCTZ = hydrochlorothiazide; QT = time between start of Q wave and end of T wave; TB = tuberculosis

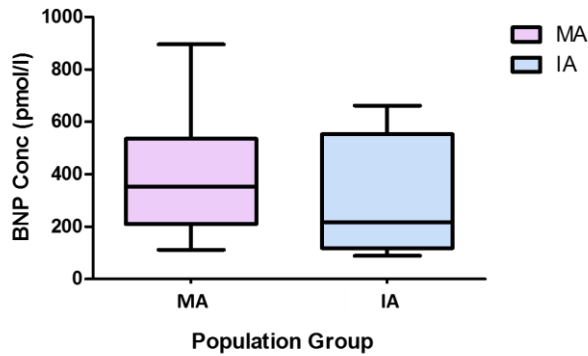
### 6.3.3 Statistical Analysis

Patients were analysed as a combined cohort as well as after stratification into population groups. The median age of this sub-cohort was 51.5 years, which, when stratified showed MA patients with a median age of 53.5 years compared to the younger IA cohort with a median age of 46.5 years. Despite the younger age of the IA cohort, their median BMI at 31.6 was higher and in the obese category compared to the median BMI of the MA cohort at 28.2 - in the overweight category.

While the IA cohort had a higher incidence of hypertension (0.5) compared to the MA cohort (0.44), the MA cohort had more pre-existing cardiac disease (0.03), diabetes (0.08) and more smokers (0.33) compared to a zero incidence of these factors in the IA cohort. These risk factors have been shown to increase the likelihood of ACT<sup>24,26,36</sup>. This would essentially translate to the MA cohort being at higher risk of cardiotoxicity and therefore likely to have higher expected levels of BNP. However, the median baseline LVEF for the combined, MA and IA cohort was 60% which is in the normal or healthy heart range<sup>64</sup>.

Cumulative chemotherapeutic dose relies on type and stage of cancer as well as the BSA which relies on both weight and height<sup>184</sup>. While BMI is a crude but relatable measure, the IA cohort with a higher median BMI received overall, a higher median cumulative dose of doxorubicin – i.e. 440mg/m<sup>2</sup> compared to the MA cohort with a median dose of 400mg/m<sup>2</sup> in this pilot biomarker cohort. Only one IA patient was administered epirubicin so a similar deduction could not be made for this drug. However, when both cohorts were analysed for median dose of anthracycline-based chemotherapy, the IA sub-cohort received a higher dose – 445mg/m<sup>2</sup> compared to the MA cohort's cumulative median dose of 400mg/m<sup>2</sup>.

While the increased concentration of BNP is associated with CF, a major limitation of this measure is that BNP levels may be sensitive to other factors such as patient age, gender and obesity, as well as population-specific variants resulting in increased sensitivity to chemotherapy<sup>127,139,140</sup>. Therefore, BNP baseline levels were stratified according to population group (Fig.45).



**Figure 45: Comparison of baseline BNP concentrations in both the MA and IA cohorts with the exclusion of all hypertensive patients (n=40, p=0.4369, Unpaired t-test)**

Hypertensive patients were excluded from this analysis as hypertension may cause ventricular stretching which can cause increases in BNP levels (Fig. 45, Table 41) <sup>175</sup>.

**Table 41: Comparison of median baseline BNP concentrations before the administration of anthracycline based chemotherapy and stratified per the type of chemotherapy administered, population group and hypertensive status of patient**

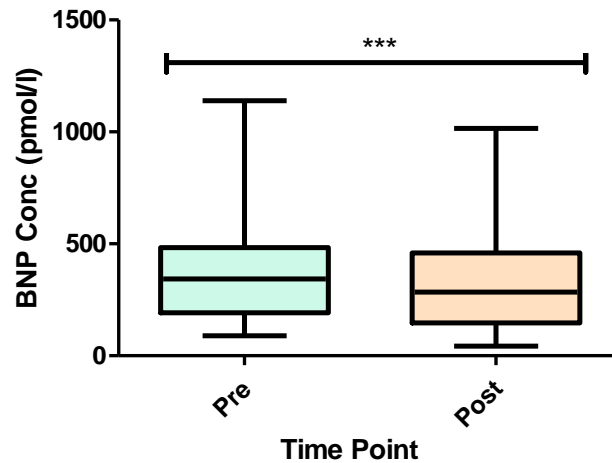
Patient Cohort	Type of Anthracycline	BNP Concentration (mg/m <sup>2</sup> )		Population Group	p-value
		Median	Range		
All	Doxorubicin	344.4	93.7-1139.8	MA	<0.0001*
		362.3	206.5-662	IA	
	Epirubicin	343.3	187.1-895.8	MA	
		89.5 <sup>^</sup>	n/a	IA	
	Both	344.4	93.7-1139.8	MA	
		295.1	89.5-662	IA	
Hypertensive	Doxorubicin	290	93.7-1139.8	MA	<0.0001*
		375.3	362.3-388.2	IA	
	Epirubicin	324.2	187.1-362.3	MA	
		89.5 <sup>^</sup>	n/a	IA	
	Both	307.1	93.7-1139.8	MA	
		362.3	89.5-388.2	IA	
Not Hypertensive	Doxorubicin	344.9	112.4-738.4	MA	-+
		227.9	227.9-662	IA	
	Epirubicin	366.3	218.8-895.8	MA	
		n/a	n/a	IA	
	Both	354.1	112.4-895.8	MA	
		227.9	227.9-662	IA	

\*Chi-square Test, +Not computed due to no IA, non-hypertensive patients administered Epirubicin, p-value<sub>both</sub> <0.0001 therefore statistically significant, <sup>^</sup>Only one Indigenous African patient was administered epirubicin

Overall, hypertensive IA patients had higher baseline BNP levels compared to their MA counterparts. However, MA patients (both hypertensive and non-hypertensive

combined) had higher baseline BNP concentrations compared to IA patients (Table 41). Chi-square test analysis shows that this difference is statistically significant ( $p_{\text{overall}} < 0.0001$ ).

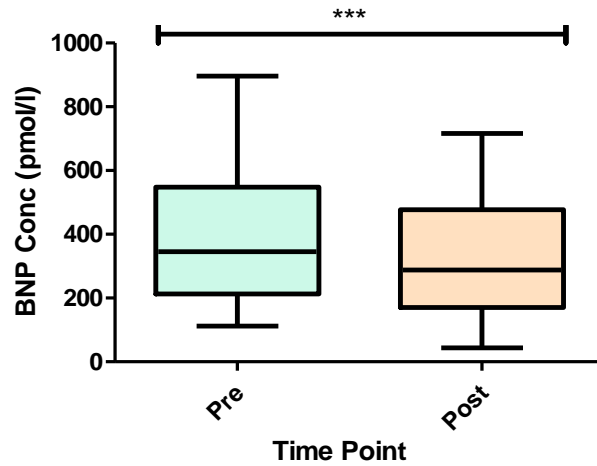
The BNP concentration of the entire cohort before and after chemotherapy is illustrated in Figure 46.



**Figure 46: BNP Concentration of the entire cohort before (pre) and after (post) chemotherapy (n=42), ( $p < 0.001$ , Chi-square Test)**

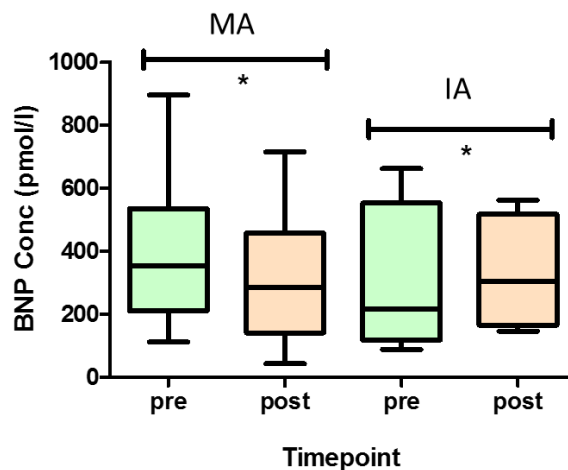
While the differences in BNP levels for the combined populations (pre vs post, both hypertensive and non-hypertensive) are statistically significant, Figure 46 indicates that anthracycline-based chemotherapy decreases BNP concentration after administration, which is counterintuitive. Anthracycline-based chemotherapy is known to be cardiotoxic and was therefore expected to increase BNP levels rather than show a decrease. Therefore, the BNP concentration of the entire cohort was re-analysed, excluding the hypertensive patients, in case the decreased BNP levels after chemotherapy is as a result of the use of anti-hypertensives<sup>185,186</sup>.

The BNP concentration of the non-hypertensive patients in the cohort were compared before and after chemotherapy and illustrated in Figure 47.



**Figure 47: BNP Concentration of the non-hypertensive patients in the cohort before and after chemotherapy (n=21), ( $p < 0.001$ , Chi-square Test)**

Despite the exclusion of hypertensive patients, there was still a uniform decrease in BNP levels after the administration of chemotherapy (Fig. 47). This shows that this trend is not due to hypertension and the use of anti-hypertensives. Therefore, as previously discussed (Fig. 45), stratification of the cohort into population group before the analysis of BNP concentration levels may allow for a more accurate analysis as seen in Figure 48.

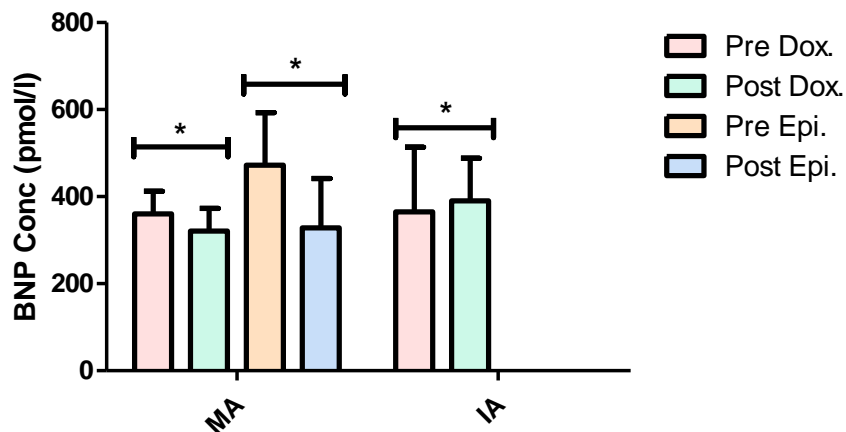


**Figure 48: Comparison of BNP levels at baseline (pre) and after (post) chemotherapy for both MA and IA patients with the exclusion of hypertensive patients ( $p < 0.0001$  statistically significant change from baseline to after chemotherapy, chi-square test for both MA and IA)**

Figure 48 demonstrated a statistically significant change from baseline to after chemotherapy in BNP concentration levels in both populations. While BNP levels decreased in the MA population after the administration of chemotherapy as it has

with the previous analyses (Figs.45 & 48), it shows a posttreatment increase in the IA cohort, indicating possible quantifiable cardiac dysfunction (Fig. 49). Furthermore, this pilot analysis shows that BNP levels may be ethnicity dependent and that the anthracycline-based chemotherapy has potential cardiotoxic effect in the IA population.

This effect was interrogated further whereby pre- and post-treatment measures were analysed taking account of the type of anthracycline administered in each of the MA and IA ethnic groups.



**Figure 49: Comparison of BNP levels at baseline and after chemotherapy for both MA and IA patients with the exclusion of hypertensive patients and stratified into type of anthracycline-based chemotherapy administered**

Within the MA cohort, BNP levels were found to be significantly different (i.e. decreased) from baseline to post-chemotherapy in patients treated with doxorubicin and as well as in patients treated with epirubicin ( $p < 0.0001$ , Chi-Square Test, Fig. 49). In this MA cohort, there is a trend toward BNP levels decreasing after treatment with doxorubicin. The MA patients scheduled for epirubicin had a higher BNP concentration at baseline than the patients scheduled for doxorubicin.

Similarly, within the IA cohort, BNP levels were found to be significantly different from the baseline time-point to the time-point after the administration of chemotherapy in patients treated with doxorubicin ( $p < 0.0001$ , Chi-Square Test, Fig. 48). IA patients scheduled for doxorubicin had slightly higher baseline BNP levels compared to their MA counterparts. This observation is interesting given that the IA cohort had a lower clinical risk in terms of co-morbidities (Table 41). The trend for doxorubicin is an increase of BNP levels after the administration of treatment indicating that IA may be more susceptible to cardiotoxic chemotherapy as previously inferred to (Fig. 48).

Because of the small sample size – this observation does need to be interpreted with caution.

While the assessment of BNP levels at specific time-points (pre- vs post chemotherapy) is of value, it is important to correlate these levels with patient clinical status in order to ascertain if BNP concentrations are indicative of cardiac physiological insult or dysfunction.

### 6.3.4 Correlation between Routine Measures, Biomarkers and Cardiac Function Decline

As previously mentioned, a severity score was established where patients were stratified in terms of anthracycline-induced cardiotoxicity. The score was based on the Common Technology Criteria for Adverse Events v3.0 (CTCAE) which has been utilised previously<sup>7,10</sup>. This score or grading was based on change in LVEF if available and clinical phenotype after the administration of chemotherapy.

**Table 42: Severity Score established using LVEF measure and patient clinical phenotype**

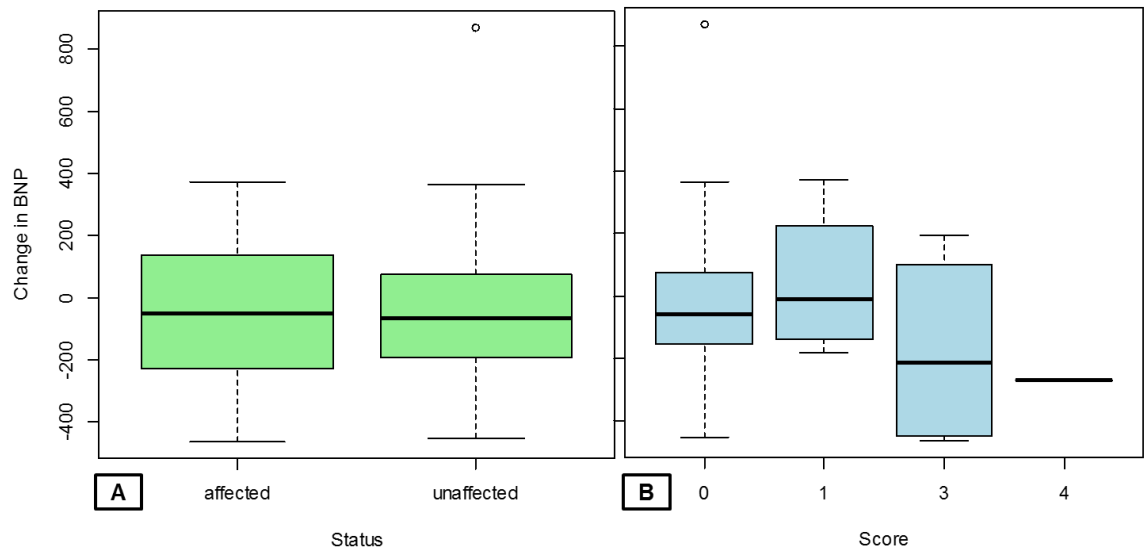
Severity Score	Change in LVEF	Clinical Phenotype
0	No change	Normal
1	No LVEF decline	Chest pain, shortness of breath, palpitations
2	LVEF decline <10%	Chest pain, shortness of breath, palpitations, chemotherapy dose change or decrease
3	LVEF decline <10%	Suspected CCF, LV Dilation, Cardiomegaly, raised Troponin T
4	LVEF decline ≥ 10%	Congestive Heart Failure, Myocardial Infarction

Due to the progressive nature of cardiac dysfunction, it was necessary to take certain clinical characteristics beyond LVEF into account<sup>51-54</sup>.

Cumulatively (in both population groups), the change in BNP concentration levels (post – pre) was not normally distributed ( $W=0.938$ ;  $p=0.029$ ). The median BNP change is -59 pmol/l indicating that there was an overall average decrease of 59 pmol/l from pre-chemotherapy levels to post-chemotherapy levels. In terms of the extremes in BNP levels change – this ranged from a decrease of 463.2 pmol/l to an increase of 869.6 pmol/l.

The change in BNP was analysed in terms of clinical status of the patient after the conclusion of chemotherapy and the following emerged: despite there being evidence

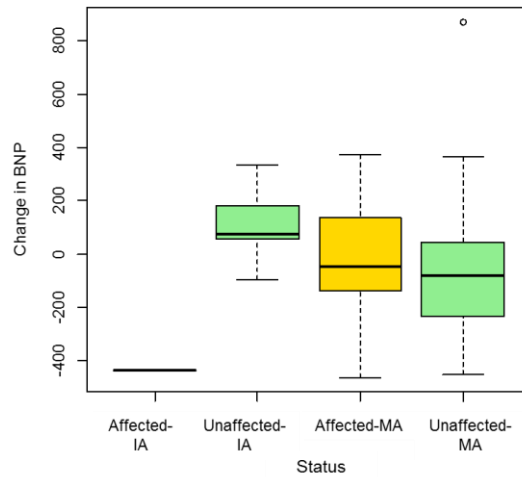
of the patient having some measure of cardiac dysfunction, there was no statistically significant difference in change in BNP levels between the unaffected and affected groups (Fig. 50a, Chi-square test,  $p=0.426$ ).



**Figure 50: Comparison of change in BNP based on both patient status (A) and severity score (B)**

Figure 50A demonstrates that despite the clinical status of patients after treatment, there is no difference in change in BNP levels. Figure 50B shows that while there are differences depending on the severity score, these differences indicate that BNP levels decrease in patients with a higher severity score (i.e. BNP levels after chemotherapy have a greater decrease in patients with signs of cardiac dysfunction) which is counterintuitive.

Patients were then stratified into population group and assessed for change in BNP compared to their cardiac clinical status (affected vs unaffected).



**Figure 51: Comparison of change in BNP in both IA and MA affected (yellow) and unaffected (green) individuals**

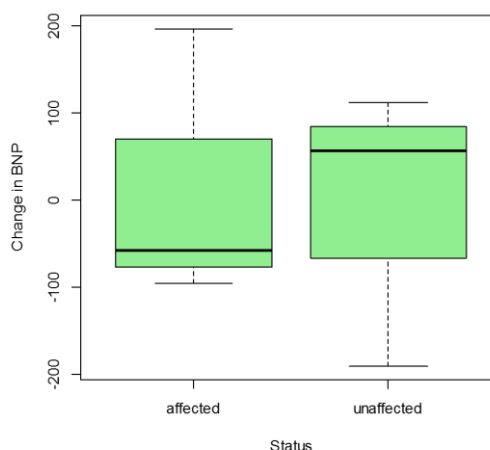
While there is no discernible difference in the change in BNP between affected and unaffected MA individuals, there is a difference between affected and unaffected IAs (Fig. 51).

Furthermore, patients with baseline cardiac incidental findings (Table 43) were analysed together with their levels of BNP and clinical status.

**Table 43: Patients with baseline cardiac incidental findings analysed for both BNP levels and clinical status**

<u>Patient ID</u>	<u>Pre LVEF</u>	<u>Cardiac Baseline</u>	<u>HPT</u>	<u>BNP</u>			<u>Score</u>	<u>Status</u>
				<u>Pre</u>	<u>Post</u>	<u>Change</u>		
ACT223	55	LVH	Y	89.5	146.0	56.5	0	unaffected
ACT232	55	Impaired relaxation LV	N	218.8	415.3	196.5	0	affected
ACT234	55	Impaired relaxation, elevated atrial filling pressures	N	176.2	288.1	111.8	0	unaffected
ACT238	60	Impaired relaxation LV, aortic regurgitation	N	593.6	402.6	-191.1	0	unaffected
ACT251	60	Tricuspid regurgitation	Y	187.1	129.3	-57.8	0	affected
ACT260	55	LVH	N	247.2	151.9	-95.3	1	affected

Table 43 shows patients with both baseline cardiac incidental findings and measurement of BNP levels before and after chemotherapeutic administration – despite potential diminished cardiac function, increases in BNP levels were not indicative of clinical status. This lack of association was illustrated in Figure 52.



**Figure 52: BNP changes in patients with baseline cardiac incidental findings compared to their clinical status after the administration of chemotherapy**

Chi-square test for change in BNP levels compared to status (Fig. 52) showed non-significant differences ( $p=0.3062$ ).

## 6.4 Discussion

Given the inadequacy of LVEF, this prompted the validation and utilisation of BNP concentration as a measure of cardiac dysfunction and to improve the accuracy of gauging the cardiac phenotype<sup>62,93,165</sup>. Ideally, the entire cohort would have undergone BNP analysis, however, the combined cost of 272 patients at two time points limited the study to prioritisation of those without a second LVEF measure; so at best the present study is a pilot.

Data derived from our cohort of 42 patients, treated for breast cancer, indicated that, in general, BNP levels decrease after the administration of a cardiotoxic treatment. This is of interest as it can be erroneously inferred that anthracyclines may improve cardiac function or, more rationally, that the measurement of BNP concentration as a surrogate marker of cardiac function is limited. However, this trend persisted even after the removal of hypertensive patients (as BNP may be released in response to ventricular stretching of the myocardium<sup>175</sup>), as well as when the data was further stratified into the type of regimen (i.e. doxorubicin versus epirubicin). It was found that there was a discernible and significant increase in BNP concentration for the IA cohort – indicating that despite the limitation of BNP levels, the cardiac effect of the anthracyclines may have been sufficient to show impairment in this population group. Although the IA cohort was relatively small, this trend may suggest that African women may be more sensitive to chemotherapy which is plausible considering previous

evidence of ethnically variant polymorphisms that influence doxorubicin pharmacokinetics and pharmacodynamics <sup>127,139</sup>.

While the lowered levels of BNP after the administration of cardiotoxic chemotherapy, was unexpected – numerous studies have also found conflicting results correlating BNP and/or NT-proBNP levels with chemotherapy <sup>187-190</sup>. This may be explained by the difference in BNP versus Troponin – Troponin is released due to cardiac myocyte damage and/or death whereas BNP is released due to stretching of the ventricular myocardium <sup>191,192</sup>. Additionally, maintained preload reserve may explain the unchanged or decreased BNP levels after the administration of chemotherapy – compensatory mechanisms that preserve stroke volume and cardiac output thereby preventing excess expansion and pressure overload and subsequent release of BNP may explain this phenomenon <sup>186</sup>.

BNP levels are not only sensitive to functional genetic variants which may be population specific, but also to patient age, gender, obesity and hypertension <sup>127,139,140</sup>. While hypertensive patients may exhibit higher levels of BNP due to ventricular stretching and therefore not be as informative as a predictor of cardiac dysfunction, hypertensive medications such as angiotensin inhibitors and  $\beta$ -blockers, may preserve cardiac function particularly in patients treated with a sufficiently high dose of the drug <sup>69,185</sup>. It is hypothesized that the progressive nature of myocardial injury enabling the asymptomatic cardiac decline to become irreversible, symptomatic cardiac dysfunction may be slowed or even halted by the concomitant administration of anti-hypertensive medications <sup>185</sup>.

It is also likely that patients may not have left ventricular remodelling, causing systolic dysfunction, thereby not showing an increase in BNP levels. There was a difference in IA patients (Fig.51), which indicated that clinically unaffected (i.e. relating to CF) individuals have higher BNP levels after chemotherapy compared to baseline. Against this background - the one affected individual who had lower BNP levels after chemotherapy compared to before the administration of chemotherapy – suggests that BNP levels are not indicative of cardiac dysfunction but could point to ventricular stretching without cardiomyocyte death. However, we cannot exclude ACT related to cardiomyocyte death using BNP levels which is more closely related to cardiac stress and contractile dysfunction than cardiomyocyte death, as previously discussed.

While the correlation of BNP levels to patient clinical status proved to be inconclusive, the severity score, developed for increased accuracy in patient phenotyping also failed to show an association with BNP levels, suggesting that the BNP biomarker

may not be an early predictor of ACT. Furthermore, the cardiac incidental findings at baseline were compared to BNP levels and also showed no association despite there being quantifiable cardiac compromise.

#### **6.4.1 Study Limitations:**

Limitations related to the use of BNP analysis for the assessment of cardiac function include:

i) lack of two LVEF measures for every patient – despite the insensitivity of the LVEF measure in some instances, the lack of a second measure complicated the correlation of BNP measures to cardiac function;

ii) attributing differences in biomarker levels to self-reported ethnicity – which may decrease population-specific significance . Ancestry Informative markers may have been useful in delineating the different population groups rather than the reliance on self-reporting <sup>164</sup>, and

iii) lack of biomarker analysis for the entire cohort – although BNP had limited utility, data on the entire cohort may have provided more useful insight.

iv) the use of only one blood sample at each time-point due to the patients in the study being outpatients, whereas previous studies used serially collected blood samples to determine BNP concentration <sup>190</sup>. Therefore, as indicated previously in literature, BNP levels may increase in response to chemotherapy (first 72 hours) but as the damage may be transient, will then decrease and return to a “normal” range compared to persistently high BNP levels only being present in patients with irreversible cardiac dysfunction <sup>187,190</sup>;

v) The haemolysis of some patient samples were then looked at as a reason for the exaggerated baseline BNP levels. Haemolysis of a blood sample is defined as the pink or red colour of plasma or serum after centrifugation, specifically a free haemoglobin concentration of more than 0.3g/L <sup>182</sup>. While haemolysis of blood was thought to invalidate the results of biomarker assays owing to a lag time between when peripheral blood was obtained up to its usage, more recent findings have indicated otherwise. Daves *et al.* <sup>182</sup> demonstrated that moderate haemolysis had no effect on the reliability of cardiac biomarker results. *In vitro* haemolysis occurs in approximately 3.3% of all routinely collected samples and is the leading cause of up to 70% specimen rejection, by laboratories <sup>182</sup>. It is important to quantify the effect of moderately haemolysed samples on cardiac biomarker testing as it has ramifications on the potentially unnecessary rejection of patient specimens and the clinical

management of such patients pertaining to diagnosis and treatment <sup>182</sup>. Essentially, the level of haemolysis needs to be gauged accurately so that severely haemolysed samples that may invalidate biomarker results are discarded.

vi) Epirubicin treatment has been shown to be less cardiotoxic than doxorubicin treatment <sup>2</sup>. Possibly due to the small number of patients, our sub-study failed to conclude on any treatment-type effect (epirubicin vs. doxorubicin). For instance, out of six patients in the IA cohort, only one patient was treated with epirubicin; and

vii) the lack of an echocardiographic evaluation after chemotherapy preventing the correlation of BNP levels to quantitative measure – routine clinical management of patients in a resource-poor environment does not always include a post-chemotherapy echocardiography.

#### **6.4.2 Recommendations:**

This pilot study has been inconclusive about the use of BNP as a measure of irreversible cardiac damage as a result of chemotherapy. However, there is every indication for expanding the study, especially focussing on ethnicities towards establishing valid population-relevant baseline measures, as well as potential changes and factors which influence this, including anthracycline as a chemotherapeutic. This would also be an opportunity to gauge whether Troponin, and perhaps ST2 and other biomarkers may be a better marker of early cardiotoxicity <sup>188,192</sup>. The excellent collaboration that has been established with the Department of Cardiology, here at the University of Cape Town for the preliminary study presented here, will facilitate the design of such a comprehensive study.

## Chapter 7: Concluding Remarks

Despite genotypes not emerging as a predictor of ACT in this study, the increased susceptibility of the IA population to ACT as well as increased BNP levels after chemotherapy, demands a closer look. Furthermore, the interrogation of IA patient genomes for novel variants of susceptibility to ACT are recommended; this requires building up of a substantial cohort from this population group, which would likely require collaboration with a number of South African health care institutions.

Furthermore, this study illustrates the need for clinical trials for new and existing drugs to be conducted in Africa on local populations. Both the present study and literature <sup>6,139,140</sup> show that local populations may have both increased risk of adverse drug reactions and/or reduced efficacy compared to their CA counterparts whose risk is already established in historical clinical trials.

The development of both the patient scoring system and statistical model to predict post-chemo LVEF may warrant further development and utilisation in a resource-poor setting.

Findings derived from this study indicate the need for refined patient management of ACT in a South African population to potentially allow for treatment with minimised risk and event-free breast cancer survival.

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## Appendices

### Appendix A: Gene Annotation and Primer Design Protocol

Before annotation of your genes, you will need to prepare files and your information will come from NCBI and Ensembl.

It is simplest to create a gene annotation folder, and create subfolders of each gene of interest which will contain the files specific to that gene.

#### NCBI ([www.ncbi.nih.gov](http://www.ncbi.nih.gov))

- Navigate to the NCBI website
- Select Gene under “All Databases” and type the gene name in the search box
- Select for the Homo Sapiens in your gene search results
- This will open a page with a “Summary” for the gene
- Copy and Paste the gene summary (until Genomic Context) into a Word Document and label it GeneName\_Summary and save in the specific gene folder that you already created. Save and Close
- Scroll down to Genomic Regions, Transcripts and Products and make sure the Reference Sequence Primary Assembly is selected and select Genbank where “Go to Nucleotide” is shown
- This will open a page with the gene details followed by the sequence (the selected region should be sufficient for SNP genotyping purposes)
- Copy the page from “LOCUS...” Till “//” and paste in Notepad and save in your gene folder as GeneName.seq
- The SEQ file must be edited to allow for accurate annotation later so please do the following:
  - o Note the Gene ID, the orientation of the strand and transcript ID – this information can be gathered from the NCBI gene page that you first navigated to as well as the link on that page called “SNP: GeneView”
  - o SNP:GeneView will also allow you to double check the validity of your SNPs as well as orientation and transcript ID
  - o With this information, go through the gene, mRNA, CDS, misc\_RNA info and for those with different gene and transcript IDS, insert a space...for example mRNA becomes m RNA etc. Also, spaces can be inserted to STS if any
  - o Save and close when done – this file is ready for annotation

#### Ensembl ([www.ensembl.org](http://www.ensembl.org))

- Navigate to the Ensembl website
- Under “All Species” select Human and type the gene name in the Search Box next to it
- Find the version of the gene you require and select (ie Human Gene version)
- This will open up a page where a Transcript table is visible, find the longest transcript (Note down the Gene ID (ENSG...), Transcript ID (ENST...) and orientation of strand for later)

- Select the longest transcript
- On the left hand side of the page, click on “Export Data” which will open a pop-up, select the following:
  - o Select GenBank format
  - o Select Feature Strand
  - o Select 10000 bases upstream and 10000 bases downstream
  - o Under “Options for GenBank”, select only “Variation Features”, “Marker Features” and “Gene Information”
  - o Click Next
  - o Select Text
- This will open up another page in your browser with the required information
- Select all, copy and paste into Notepad. Save the file in your gene folder as GeneName.ee.seq
- Again, this SEQ file must be edited before annotation:
  - o With the Gene and Transcript IDs noted earlier, go through the text and insert spaces to genes, mRNA, misc\_RNA that are not needed
  - o Save and Close

## PERLV5

For the PerlV5 first timer:

After installing PerlV5 on your laptop or PC

Create a PerlV5 folder in your C drive, every time you create new NCBI and Ensembl gene SEQ files, add them to this folder (after editing!)

- Click the start icon, search for cmd and select
- This opens up the command line for Windows
- For command line: cd.. will navigate you back a directory; to check what is in a directory you may type in dir
- Navigate to the C:\>
- You may check if your PerlV5 folder is there by typing dir so that it looks like this C:\>dir and press Enter
- If your PerlV5 folder shows on the list then you may proceed further
- Type in cd perl5 so that it looks like this **C:\>cd perl5**, press Enter
- Type in pre-perl so that it looks like this **C:\Perl5>pre-perl**, press Enter
- The next line will look like this **C:\Perl5>**
- You are ready to annotate!
- For NCBI:
  - o For forward strand orientation (plus strand), type the following:
    - o perl annotv9.pl gene.seq gene.annot s
    - o it will look like this: **C:\Perl5>perl annotv9.pl gene.seq gene.annot s**
    - o Press Enter and if the next line is **C:\Perl5>** then it has worked!
  - o For reverse strand orientation (minus strand), type the following:
    - o perl annotv9.pl gene.seq gene.annot sr
    - o it will look like this: **C:\Perl5>perl annotv9.pl gene.seq gene.annot sr**
    - o Press Enter and if the next line is **C:\Perl5>** then it has worked!

- For Ensembl:
  - For forward strand orientation, type the following:
  - perl annotv9ev2.pl genee.seq genee.annot s
  - it will look like this: **C:\Perl5>perl annotv9ev2.pl genee.seq genee.annot s**
  - Press Enter and if the next line is **C:\Perl5>** then it has worked!
  - For reverse strand orientation, type the following:
  - perl annotv9ev2.pl genee.seq genee.annot sr
  - it will look like this: **C:\Perl5>perl annotv9ev2.pl genee.seq genee.annot sr**
  - Press Enter and if the next line is **C:\Perl5>** then it has worked!

## **Appendix B: Informed Consent**

### **Consent Document**

Consent to Participate in Research:

Good Day Madam/Sir

Prof Raj Ramesar (Human Genetics, UCT), A/Prof H. Simonds (Radiation Oncology, Tygerberg Hospital) and Miss H.Naidoo (Human Genetics, UCT) will be the researchers on this study. Miss H.Naidoo will be using this research project to obtain her Doctorate.

This study will focus on damage caused to the heart by chemotherapy and its genetic basis.

You have been asked to participate in this research study by allowing us access to two tubes of your blood to run tests on and allow us access to your medical history so that we may monitor your response to treatment. Your samples will be stored securely in the Human Genetics Laboratory with access only to the investigators for a period of 3 years. At the end of the study, all samples will be destroyed. Patient samples will not be used for other studies unless new ethical approval is applied for and obtained.

**Your HIV status will be made available only to the researchers and will be kept confidential.**

Your personal and medical details will be kept confidential and you will remain anonymous in the study. You will not be at any risk by being part of this study and will not need to pay any money for the additional laboratory tests. One extra tube of blood at diagnosis and again after 3 cycles of chemotherapy will be taken with your routine blood draw at no extra risk. The unlikely risks of any blood draw include: pain, infection, bruising and minor bleeding after blood is taken.

Your participation in this research is voluntary and you will not be given any compensation for your involvement in the study. Furthermore, you will not be penalized or lose benefits if you refuse to participate or decide to stop at any time. If you agree to participate, you will be given a signed copy of this document and information about the research.

The benefit of this study will be information on the genes responsible for damage to the heart when cancer patients receive chemotherapy.

You have been informed about the study by.....

You may contact Horacia Naidoo at 021 406 6501 at any time if you have questions about the research. You may also contact the Human Research Ethics Committee if you have any concerns or queries on 021 406 6338 or email [sumayah.ariefdien@uct.ac.za](mailto:sumayah.ariefdien@uct.ac.za).

The research study, including the above information, has been described to me orally. I understand what my involvement in the study means and I voluntarily agree to participate. I have been given an opportunity to ask any questions that I might have about participation in the study.

Please tick if you agree to allow us to run more tests on your blood samples \_\_\_\_\_

Please tick if you agree to allow us access to your medical files \_\_\_\_\_

.....  
Signature of Participant Date

.....  
Signature of Translator (if applicable) Date

.....  
Signature of Witness Date

## Appendix C: Ethics Approval – UCT

UNIVERSITY OF CAPE TOWN



Faculty of Health Sciences  
Faculty of Health Sciences Human Research Ethics Committee  
Room E52-24 Groote Schuur Hospital Old Main Building  
Observatory 7925  
Telephone [021] 406 6338 • Facsimile [021] 406 6411  
e-mail: [sumayah.ariefeldien@uct.ac.za](mailto:sumayah.ariefeldien@uct.ac.za)  
[www.health.uct.ac.za/research/humanethics/forms](http://www.health.uct.ac.za/research/humanethics/forms)

08 February 2013

HREC REF: 650/2012

Ms H Naidoo  
c/o Prof R Ramesar  
Human Genetics  
FHS

Dear Ms Naidoo

**PROJECT TITLE: THE GENETICS OF ANTHRACYCLINE-INDUCED CARDIOTOXICITY IN CANCER PATIENTS**

Thank you for addressing the issues raised by the committee.

It is a pleasure to inform you that the HREC has formally approved the above mentioned study.

**Approval is granted for one year till the 28 February 2014.**

Please submit a progress form, using the standardised Annual Report Form, if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

**Please quote the REC. REF in all your correspondence.**

Yours sincerely

A handwritten signature in black ink, appearing to be 'M. Blockman', written over a light blue horizontal line.

**PROFESSOR M. BLOCKMAN**  
**CHAIRPERSON, HSF HUMAN ETHICS**

Federal Wide Assurance Number: FWA00001637.  
Institutional Review Board (IRB) number: IRB00001938

«Ariefeldien

## Appendix D: Ethics Approval – GSH



**GROOTE SCHUUR HOSPITAL**  
Enquiries: Dr Bhavna Patel  
E-mail : [Bhavna.Patel@westerncape.gov.za](mailto:Bhavna.Patel@westerncape.gov.za)

Ms Horacia Naidoo  
C/o Dr Hannah Simonds  
Radiation Oncology Department  
LE33 – New Main Building

E-mail: [Hannah.simonds@uct.ac.za](mailto:Hannah.simonds@uct.ac.za) & [raj.ramesar@uct.ac.za](mailto:raj.ramesar@uct.ac.za)

Dear Ms Naidoo

**RESEARCH: The Genetics of Anthracycline-induced Cardiotoxicity in Cancer Patients**

Your recent letter to the hospital refers.

You are hereby granted permission to proceed with your research.

Please note the following:

- a) Your research may not interfere with normal patient care
- b) Hospital staff may not be asked to assist with the research.
- c) No hospital consumables and stationary may be used.
- d) **No patient folders may be removed from the premises or be inaccessible.**
- e) Please introduce yourself to the person in charge of an area before commencing.
- f) Please discuss the study with the Head of Radiation Oncology before commencing.
- g) Confidentiality must be maintained at all times.

I would like to wish you every success with the project.

Yours sincerely

A handwritten signature in cursive script that reads 'B Patel'.

**DR BHAVNA PATEL**  
**SENIOR MANAGER: MEDICAL SERVICES**  
**Date:** 15 February 2013

C.C. Dr B. Eick  
Professor R. Abratt

G46 Management Suite, Old Main Building,  
Observatory 7925

Private Bag X,  
Observatory, 7935

## Appendix E: Ethics Approval – SUN/TBH



UNIVERSITEIT • STELLENBOSCH • UNIVERSITY  
Jou kennisvennoot • your knowledge partner

### Approval Notice New Application

25-Mar-2015  
Naidoo, Horacia H

**Ethics Reference #:** S15/02/032

**Title:** The genetics of anthracycline-induced cardiotoxicity in cancer patients.

Dear Ms Horacia Naidoo,

The **New Application** received on **27-Feb-2015**, was reviewed by members of **Health Research Ethics Committee 2** via Expedited review procedures on **18-Mar-2015** and was approved.

Please note the following information about your approved research protocol:

Protocol Approval Period: **25-Mar-2015 -25-Mar-2016**

Please remember to use your **protocol number** (S15/02/032) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

#### **After Ethical Review:**

Please note a template of the progress report is obtainable on [www.sun.ac.za/rds](http://www.sun.ac.za/rds) and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372  
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

#### **Provincial and City of Cape Town Approval**

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health ([healthres@pgwc.gov.za](mailto:healthres@pgwc.gov.za) Tel: +27 21 483 9907) and Dr Helene Visser at City Health ([Helene.Visser@capetown.gov.za](mailto:Helene.Visser@capetown.gov.za) Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required **BEFORE** approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and documents please visit: [www.sun.ac.za/rds](http://www.sun.ac.za/rds)

## **Appendix F: Reagent Preparation**

### **Primer Preparation:**

#### 1x TE Buffer:

10mM Tris added to 1mM EDTA at pH 8.0 makes up a 10x TE Buffer stock solution. A dilution of 1:10 with distilled water will produce 1x TE Buffer.

### **DNA Isolation:**

#### Red Blood Cell Lysis Buffer (RBC Lysis Buffer):

8.28g of Ammonium Chloride (NH<sub>4</sub>Cl), 079g of Ammonium Bicarbonate powder (NH<sub>4</sub>HCO<sub>3</sub>), 0.2ml of EDTA (0.5M, pH 7.4) made up to 1L with distilled water.

##### - 0.5M EDTA:

18.612g of EDTA in 60ml of dH<sub>2</sub>O, adjust pH to 7.4 and make up to total volume of 100ml.

#### Cell Lysis Buffer:

25ml of Tris-HCl (1M, pH 7.5), 16.7ml of 3M NaCl and 1ml of 0.5 M EDTA made up to 500ml with distilled water.

##### - 1M Tris-HCl:

12.1g of Tris in 60ml of dH<sub>2</sub>O, adjust pH to 7.5 and make up to total volume of 100ml

##### - 3M NaCl:

17.532g of NaCl in 100ml of dH<sub>2</sub>O

##### - 0.5M EDTA:

18.612g of EDTA in 60ml of dH<sub>2</sub>O, adjust pH to 7.4 and make up to total volume of 100ml

#### 20% SDS:

2g Sodium Dodecyl Sulfate in 10ml of dH<sub>2</sub>O

#### 20mg/ml Proteinase K:

0.02g Proteinase K in 1000ul of 1mM CaOAc at pH 8

#### 6M NaCl:

35.064 g NaCl in 100ml of dH<sub>2</sub>O

**Agarose Gel Electrophoresis:**

10x Tris-Borate-EDTA (TBE) Buffer:

108g Tris, 55g Boric Acid and 7.4g EDTA (pH 8) made up to 1L using distilled water.

## Appendix G: Multiplex PCR Protocol

**Aim of the experiment:** Allow for the exponential amplification of a targeted sequence for SNaPshot Genotyping

	bp	tm	cond	Change/exp peak	
HNMT	325	55		C/A (black to green)	<b>MXA</b>
ABCC2 rs818	572			G/A (blue to green)	
RAC2	341	56		A/T (green to red)	<b>MXB</b>
NCF4	725			G/A (blue to green)	
ABCC1 rs246	595	60	Tchdwn, Gly	A/G (green to blue)	<b>MXC</b>
*ABCC2 rs172	761			A/T (green to red)	

Date:

### Preparation of Mastermix

#### Materials:

	Volume for 1 reaction (µl) – 1x	Volume for mastermix (µl) - x	Volume of Mastermix (ul) – x	Volume of Mastermix (ul) – x
Taq Reaction Buffer (5X)	5 (1X)			
dNTPs (5mM)	1 (200uM)			
MgCl <sub>2</sub> (50mM/ul)	1.5 (1mM)			
F and R primer (20uM)	0.5 each (10pmol each)			
Glycerol	0 or 0.25			
dH <sub>2</sub> O	14.4 or 14.15			
Taq Polymerase (5U/ul)	0.1 (0.02U/ul)			
DNA template (100ng/ul)	2 (100ng)			
<b>TOTAL</b>	<b>25</b>			

- DNA samples and 1 negative control (water)
- Clean bench area with biocide; have all reagents on ice
- Set up master-mix without DNA and remember to add Taq last
- Add 23µl master mix to each PCR tube or plate as well as 2µl of DNA template
- Include a **control** containing 2µl master-mix and 2µl of dH<sub>2</sub>O
- Edit the machine program: 94 °C for 3min, 94 °C for 3sec, **Tm = 55°C for MX1, 2, & 3, Tm = 58°C for MX4, Tm = 60°C for MX5, , Tm = 60°C touchdown for MX6 and Singleplex**
- 72 °C for 1min repeated for 30 cycles
- 72 °C for 10min at the end
- Set the sample volume to 25µl and Run!
- Once completed – remove samples of PCR products. Use in the designated post-PCR areas or store in the fridge appropriately. Post PCR area= Zone

### **Electrophoresis of PCR products for correct temp**

#### **Materials:**

- Loading Dye - Thermoscientific (Lot No: 00148043)
- 1x TBE Tank Buffer
- Molecular Weight Marker-Thermoscientific (Lot No: 00149428)
- NuSieve Agarose – Lonza (ME, USA) (Lot No: 0000313053)

For 3% (w/v) Agarose gel (50ml or 100ml), use 1.5g or 3g agarose respectively and mix into 50ml or 100ml 1X TBE Buffer respectively. Dissolve by microwave heating. Allow to cool, add 5ul or 10ul of SyBr Safe, pour into casting tray with a well comb and allow to solidify (1-2hr min).

Immerse gel into tank buffer. Load first well with 7ul of MWM and 3ul LD or mix 200ul lab LD with 25ul of MWM and 125ul water and store in fridge and use 7ul in gel. Load 7-8ul of PCR product onto gel. Electrophorese at 90V for 50-60minutes. For larger gels, allow for run in of samples as loading proceeds. If GoTaq Reaction buffer used then no LD needed.

#### For the analysis:

- Assess sharpness and specificity of band at different temps by visualizing under UV Doc, photograph and label.

## Appendix H: Patient DNA Isolations

**Table 1: Patient DNA Isolations at Diagnosis and after Cycle 3 of Anthracycline-based Chemotherapy**

DNA Number	Pre-treatment Sample	Post-treatment Sample	Notes
ACT 1.1ANN	848.62	150.69	
ACT 2.1FLO	908.33	591.97	
ACT 3.1FAI	286.03	249.69	
ACT 4.1SUK	285.79	69.73	
ACT 5.1JUL	1204.09	806.91	
ACT 6.1GER	332.81	616.3	
ACT 7.1VUY	535.19	198.97	
ACT 8.1 LYN	860.98	1509.22	
ACT 9.1HEN	1472.8	291.65	
ACT 10.1SHA	948.92	131.2	
ACT 11.1CHA	2357.47	310.74	
ACT 12.1NAD	101	374.3	
ACT 13.1NOM	1671.5	965.99	
ACT 14.1GAY	1517.7	331.52	
ACT 15.1BER	1269.04	379.99	
ACT 16.1BON	546.74	793.25	
ACT 17.1MON	285.05	144.67	
ACT 18.1CHA	385.35	835.29	
ACT 19.1CHE	949.22	426.34	
ACT 20.1MYM	1017.5	619.58	
ACT 21.1NON	1209.6	765.63	
ACT 22.1VER	929.33	562.76	
ACT 23.1SHI	748.93	577.21	
ACT 24.1MER	321.74		Patient deceased before sample 2 procured
ACT 25.1MAR	927.17	587.09	
ACT 26.1RHO	284.81	108.5	
ACT 27.1LUC	916.28	180.9	
ACT 28.1NOE	1155.7		Patient defaulted
ACT 29.1GAD	231.58	648.42	
ACT 30.1TAN	135.93	335.36	
ACT 31.1DOR			DNA Isolation failed for sample 1; patient defaulted for sample 2
ACT 32.1SHA	395.24	420.3	
ACT 33.1SYL	151.08		Chemotherapy regimen changed
ACT 34.1NEL	307.32	328.47	
ACT 35.1NTO	1168.9		Patient defaulted

ACT 36.1NAT	228.77	323.22	
ACT 37.1KUL	131.21		No treatment administered
ACT 38.1MAR	820.18	333.33	
ACT 39.1SUS	879.15	1078.84	
ACT 40.1MON	101.57	190.86	
ACT 41.1PHE	239.3		Patient defaulted
ACT 42.1LIE	383.96		Patient defaulted
ACT 43.1DEN	508.37	214.14	
ACT 44.1HEL	778.58	520.41	
ACT 45.1NOK	594.36	662.52	
ACT 46.1ELI	153.71	402.42	
ACT 47.1SHA	180.16	346.74	
ACT 48.1YAL	275.37		Patient defaulted
ACT 49.1NOS	260.96	44.9	
ACT 50.1PAM	452.16	103.2	
ACT 51.1BON	54.78	92.54	
ACT 52.1ANN	45.41		Patient defaulted
ACT 53.1DEB	81.21		Patient defaulted
ACT 54.1BAR	229.35	53.64	
ACT 55.1MAR	332.43		Patient defaulted
ACT 56.1FEL	55.2		Patient defaulted
ACT 57.1OLI	24.96	73.5	
ACT 58.1DOR	78.4		Patient defaulted
ACT 59.1JAN	28.3	149.09	
ACT 60.1MAR	155.93	54.8	
ACT 61.1LAT	102.7	120.5	
ACT 62.1THA	65.6	57.6	
ACT 63.1ELI	25.1	68.8	
ACT 64.1NOK	29.8	147.7	
ACT 65.1MAR	88.62	52.9	
ACT 66.1RAC	162.72	154.2	
ACT 67.1TES	356.67	51.1	
ACT 68.1MAR	308.62	87.4	
ACT 69.1NOB	118.7		Patient defaulted
ACT 70.1KAR	193.1	139.02	
ACT 71.1SHI	147.3		Sample 2 stored as buffy
ACT 72.1MON	49.6		Sample 2 stored as buffy
ACT 73.1ALY	65.3		Sample 2 stored as buffy
ACT 74.1RAG	176.01		Sample 2 stored as buffy
ACT 75.1ZUK	56.42		Sample 2 stored as buffy
ACT 76.1DEL	138.23		Sample 2 stored as buffy
ACT 77.1CHR	190.57		Sample 2 stored as buffy
ACT 78.1BON	95.6		Sample 2 stored as buffy
ACT 79.1VER	82.86		Sample 2 stored as buffy
ACT 80.1MAR	169.89		Sample 2 stored as buffy

ACT 81.1ELI	248.69		Sample 2 stored as buffy
ACT 82.1PAT	69		Sample 2 stored as buffy
ACT 83.1YVO	302.16		Sample 2 stored as buffy
ACT 84.1EUA	67.21		Sample 2 stored as buffy
ACT 85.1CHR	82.11		Sample 2 stored as buffy
ACT 86.1YOL	231.57		Sample 2 stored as buffy
ACT 87.1JOL	103.35		Sample 2 stored as buffy
ACT 88.1TAN	225.67		Sample 2 stored as buffy
ACT 89.1MAR	171.46		Sample 2 stored as buffy
ACT 90.1ANT	550.46		Sample 2 stored as buffy
ACT 91.1LIZ	340.34		Sample 2 stored as buffy
ACT 92.1JAM	118.76		Sample 2 stored as buffy
ACT 93.1NAZ	71.24		Sample 2 stored as buffy
ACT 94.1AVR	47.61		Sample 2 stored as buffy
ACT 95.1VUY	183.76		Sample 2 stored as buffy
ACT 96.1FAG	66.63		Sample 2 stored as buffy
ACT 97.1MAG	29.89		Sample 2 stored as buffy
ACT 98.1AVR	21.94		Sample 2 stored as buffy
ACT 99.1PAT	27.78		Sample 2 stored as buffy
ACT 100.1 NOM	52		Sample 2 stored as buffy
ACT 101.1MAR	177.93		Sample 2 stored as buffy
ACT 102.1PAU	286.96		Sample 2 stored as buffy
ACT 103.1INS	75.08		Sample 2 stored as buffy
ACT 104.1NOT	57.78		Sample 2 stored as buffy
ACT 105.1NAN	58.26		Sample 2 stored as buffy
ACT 106.1ELL	19.51		Sample 2 stored as buffy
ACT 107.1NOM	20.02		Sample 2 stored as buffy
ACT 108.1CHR	72.82		Sample 2 stored as buffy
ACT 109.1SAN	40.38		Sample 2 stored as buffy
ACT 110.1JEA	150.19		Sample 2 stored as buffy
ACT 111.1THE	25.68		Sample 2 stored as buffy
ACT 112.1RUB	189.17		Sample 2 stored as buffy
ACT 113.1JEA	117.57		Sample 2 stored as buffy
ACT 114.1PAM	33.72		Sample 2 stored as buffy
ACT 115.1ANN	23.25		Sample 2 stored as buffy
ACT 116.1SHI	28.37		Sample 2 stored as buffy
ACT 117.1VIO	62.99		Sample 2 stored as buffy
ACT 118.1GLE	133.19		Sample 2 stored as buffy
ACT 119.1ROW	116.77		Sample 2 stored as buffy
ACT 120.1VAL	93.94		Sample 2 stored as buffy
ACT 121.1MAR	91.54		Sample 2 stored as buffy
ACT 122.1GRA	59.51		Sample 2 stored as buffy
ACT 123.1LUL	50.23		Sample 2 stored as buffy
ACT 124.1NCU	186.94		Sample 2 stored as buffy
ACT 125.1SHA	105.63		Sample 2 stored as buffy

ACT 126.1GEO	67.88		Sample 2 stored as buffy
ACT 127.1AME	11.15		Sample 2 stored as buffy
ACT 128.1CHR	176.37		Sample 2 stored as buffy
ACT 129.1AME	111.99		Sample 2 stored as buffy
ACT 130.1MIN	121.62		Sample 2 stored as buffy
ACT 131.1ELI	102.25		Sample 2 stored as buffy
ACT 132.1NIC	89.01		Sample 2 stored as buffy
ACT 133.1AMA	153.66		Sample 2 stored as buffy
ACT 134.1ADR	103.7		Sample 2 stored as buffy
ACT 135.1FIO	107		Sample 2 stored as buffy
ACT 136.1JUR	93.76		Sample 2 stored as buffy
ACT 137.1CYN	95.41		Sample 2 stored as buffy
ACT 138.1JEA	44.63		Sample 2 stored as buffy
ACT 139.1HEN	74.48		Sample 2 stored as buffy
ACT 140.1URS	32.27		Sample 2 stored as buffy
ACT 141.1MAR	42.28		Sample 2 stored as buffy
ACT 142.1ALE	115.33		Sample 2 stored as buffy
ACT 143.1DES	102.58		Sample 2 stored as buffy
ACT 144.1NOK	43.19		Sample 2 stored as buffy
ACT 145.1JAC	110.64		Sample 2 stored as buffy
ACT 146.1ELI	52.1		Sample 2 stored as buffy
ACT 147.1SHI	158.18		Sample 2 stored as buffy
ACT 148.1GER	76.9		Sample 2 stored as buffy
ACT 149.1ZEL	287.11		Sample 2 stored as buffy
ACT 150.1KAT	47.43		Sample 2 stored as buffy
ACT 151.1AMA	94		Sample 2 stored as buffy
ACT 152.1ALE	40.94		Sample 2 stored as buffy
ACT 153.1SOF	240.04		Sample 2 stored as buffy
ACT 154.1SHA	18.09		Sample 2 stored as buffy
ACT 155.1TAN	131.21		Sample 2 stored as buffy
ACT 156.1LUC	37.16		Sample 2 stored as buffy
ACT 157.1LIL	250.23		Sample 2 stored as buffy
ACT 158.1NOM	56.89		Sample 2 stored as buffy
ACT 159.1WIL	99.1		Sample 2 stored as buffy
ACT 160.1MYR	5.17		Sample 2 stored as buffy
ACT 161.1MIC	83.91		Sample 2 stored as buffy
ACT 162.1LYN	37.72		Sample 2 stored as buffy
ACT 163.1JOH	25.27		Sample 2 stored as buffy
ACT 164.1EVA	68.35		Sample 2 stored as buffy
ACT 165.1JEN	85.04		Sample 2 stored as buffy
ACT 166.1SUS	49.55		Sample 2 stored as buffy
ACT 167.1LUN	19.95		Sample 2 stored as buffy
ACT 168.1RAC	39.83		Sample 2 stored as buffy
ACT 169.1NOK	102.67		Sample 2 stored as buffy
ACT 170.1NOV	163.38		Sample 2 stored as buffy

ACT 171.1NOK	688.71		Sample 2 stored as buffy
ACT 172.1MAR	261.47		Sample 2 stored as buffy
ACT 173.1LEA	344.45		Sample 2 stored as buffy
ACT 174.1JOA	68.97		Sample 2 stored as buffy
ACT 175.1JOH	281.45		Sample 2 stored as buffy
ACT 176.1MAR	987.31		Sample 2 stored as buffy
ACT 177.1ANN	110.75		Sample 2 stored as buffy
ACT 178.1RAC	349.14		Sample 2 stored as buffy
ACT 179.1NAZ	419.74		Sample 2 stored as buffy
ACT 180.1MIC	168.29		Sample 2 stored as buffy
ACT 181.1LAU	137.91		Sample 2 stored as buffy
ACT 182.1THA	657.25		Sample 2 stored as buffy
ACT 183.1WEN	96.84		Sample 2 stored as buffy
ACT 184.1SAR	96.31		Sample 2 stored as buffy
ACT 185.1FLO	976.93		Sample 2 stored as buffy
ACT 186.1VER	590.32		Sample 2 stored as buffy
ACT 187.1RUK	371.95		Sample 2 stored as buffy
ACT 188.1LOR	75.43		Sample 2 stored as buffy
ACT 189.1LEL	136.21		Sample 2 stored as buffy
ACT 190.1BAD	1281.4		Sample 2 stored as buffy
ACT 191.1PET	777.44		Sample 2 stored as buffy
ACT 192.1NTO	313.3		Sample 2 stored as buffy
ACT 193.1FRA	782.92		Sample 2 stored as buffy
ACT 194.1YOL	747.9		Sample 2 stored as buffy
ACT 195.1LOR	694.16		Sample 2 stored as buffy
ACT 196.1JUD	560.45		Sample 2 stored as buffy
ACT 197.1PET	183.82		Sample 2 stored as buffy
ACT 198.1ALV	192.12		Sample 2 stored as buffy
ACT 199.1PAT	106.45		Sample 2 stored as buffy
ACT 200.1KAR	440.13		Sample 2 stored as buffy
ACT 201.1NTO	66.91		Sample 2 stored as buffy
ACT 202.1JAC	196.86		Sample 2 stored as buffy
ACT 203.1BEA	155.92		Sample 2 stored as buffy
ACT 204.1MUR	22.42		Sample 2 stored as buffy
ACT 205.1ZUR	521.45		Sample 2 stored as buffy
ACT 206.1NOA	282.67		Sample 2 stored as buffy
ACT 207.1MAG	305.56		Sample 2 stored as buffy
ACT 208.1RAC	233.67		Sample 2 stored as buffy
ACT 209.1VEN	49.16		Sample 2 stored as buffy
ACT 210.1JOH	212.56		Sample 2 stored as buffy
ACT 211.1VER	440		Sample 2 stored as buffy
ACT 212.1ALI	216.81		Sample 2 stored as buffy
ACT 213.1DES	312.04		Sample 2 stored as buffy
ACT 214.1SHI	130.27		Sample 2 stored as buffy
ACT 215.1GER	555.82		Sample 2 stored as buffy

ACT 216.1FUN	646.26		Sample 2 stored as buffy
ACT 217.1MIE	97.86		Sample 2 stored as buffy
ACT 218.1PAT	271.82		Sample 2 stored as buffy
ACT 219.1MAG	468.66		Sample 2 stored as buffy
ACT 220.1MAU	199.7		Sample 2 stored as buffy
ACT 221.1BER	413.44		Sample 2 stored as buffy
ACT 222.1STE	201.95		Sample 2 stored as buffy
ACT 223.1PLI	75.69		Sample 2 stored as buffy
ACT 224.1FLO	100.25		Sample 2 stored as buffy
ACT 225.1KAR	454.62		Sample 2 stored as buffy
ACT 226.1SER	92.82		Sample 2 stored as buffy
ACT 227.1LUC	243.93		Sample 2 stored as buffy
ACT 228.1DUN	47.05		Sample 2 stored as buffy
ACT 229.1ROS	56.45		Sample 2 stored as buffy
ACT 230.1WIL	513.23		Sample 2 stored as buffy
ACT 231.1ELS	634.41		Sample 2 stored as buffy
ACT 232.1ALE	638.29		Sample 2 stored as buffy
ACT 233.1ANI	465.57		Sample 2 stored as buffy
ACT 234.1CHR	299.49		Sample 2 stored as buffy
ACT 235.1ELI	267.6		Sample 2 stored as buffy
ACT 236.1RUT	174.89		Sample 2 stored as buffy
ACT 237.1ELI	152.36		Sample 2 stored as buffy
ACT 238.1BEU	581.08		Sample 2 stored as buffy
ACT 239.1BUS	1742.5		Sample 2 stored as buffy
ACT 240.1TAR	670.17		Sample 2 stored as buffy
ACT 241.1CAT	407.82		Sample 2 stored as buffy
ACT 242.1EME	520.35		Sample 2 stored as buffy
ACT 243.1CHR	696.28		Sample 2 stored as buffy
ACT 244.1LYD	578.75		Sample 2 stored as buffy
ACT 245.1RAC	290.01		Sample 2 stored as buffy
ACT 246.1SUS	97.36		Sample 2 stored as buffy
ACT 247.1ELI	263.33		Sample 2 stored as buffy
ACT 248.1BUS	246.17		Sample 2 stored as buffy
ACT 249.1JAN	543.42		Sample 2 stored as buffy
ACT 250.1THA	89.19		Sample 2 stored as buffy
ACT 251.1WEN	600.6		Sample 2 stored as buffy
ACT 252.1JOH	306.36		Sample 2 stored as buffy
ACT 253.1FRA	369.31		Sample 2 stored as buffy
ACT 254.1JON	328.43		Sample 2 stored as buffy
ACT 255.1VAN	704.44		Sample 2 stored as buffy
ACT 256.1CAR	507.32		Sample 2 stored as buffy
ACT 257.1ANN	705.13		Sample 2 stored as buffy
ACT 258.1BER	497.99		Sample 2 stored as buffy
ACT 259.1CAR	611.26		Sample 2 stored as buffy
ACT 260.1NAO	257.88		Sample 2 stored as buffy

ACT 261.1FLO	570.03		Sample 2 stored as buffy
ACT 262.1LEZ	413.59		Sample 2 stored as buffy
ACT 263.1SUZ	59.54		Sample 2 stored as buffy
ACT 264.1ANN	454.65		Sample 2 stored as buffy
ACT 265.1EST	406.05		Sample 2 stored as buffy
ACT 266.1SAR	380.05		Sample 2 stored as buffy
ACT 267.1JOH	197.99		Sample 2 stored as buffy
ACT 268.1CAT	503.42		Sample 2 stored as buffy
ACT 269.1HAZ	489.64		Sample 2 stored as buffy
ACT 270.1NOM	439.12		Sample 2 stored as buffy
ACT 271.1MOI	657.32		Sample 2 stored as buffy
ACT 272.1MAR	232.58		Sample 2 stored as buffy
ACT 273.1THE	503.84		Sample 2 stored as buffy
ACT 274.1MER	1871		Sample 2 stored as buffy
ACT 275.1CAR	317.49		Sample 2 stored as buffy

## Appendix I: Spectrophotometric quantitation of DNA

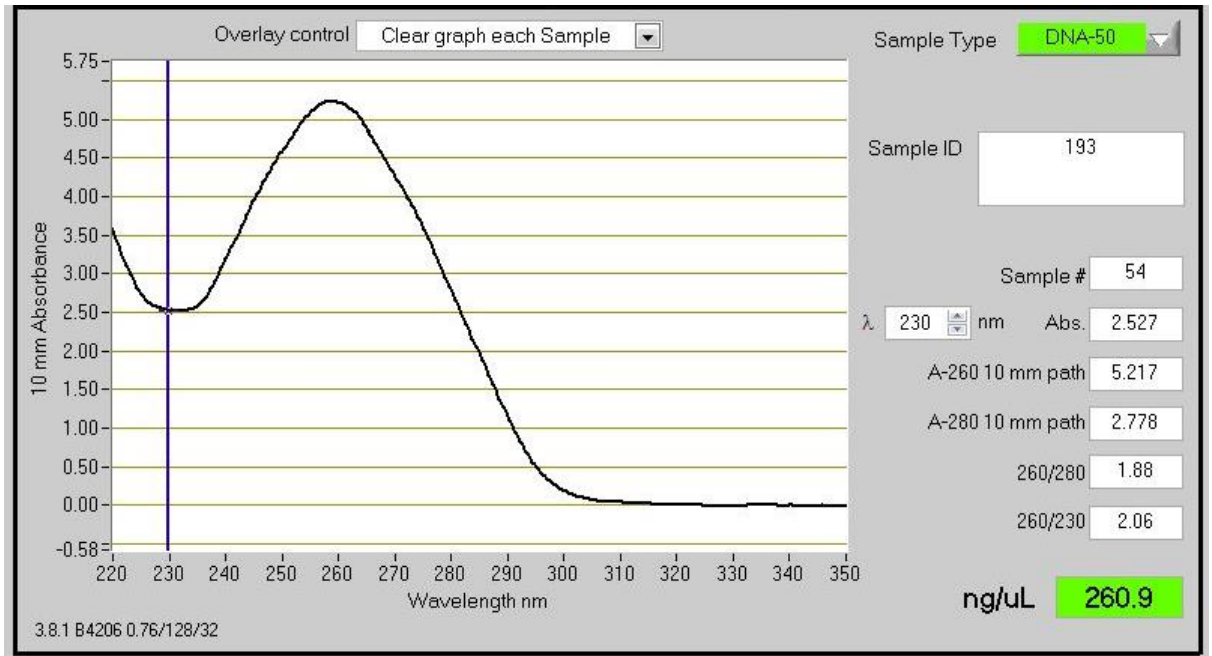


Figure 1: Representation of the spectrophotometric quantitation of patient DNA samples (ACT 193.1FRA) using the NanoDrop ND-1000 (Nanodrop Technologies, USA)

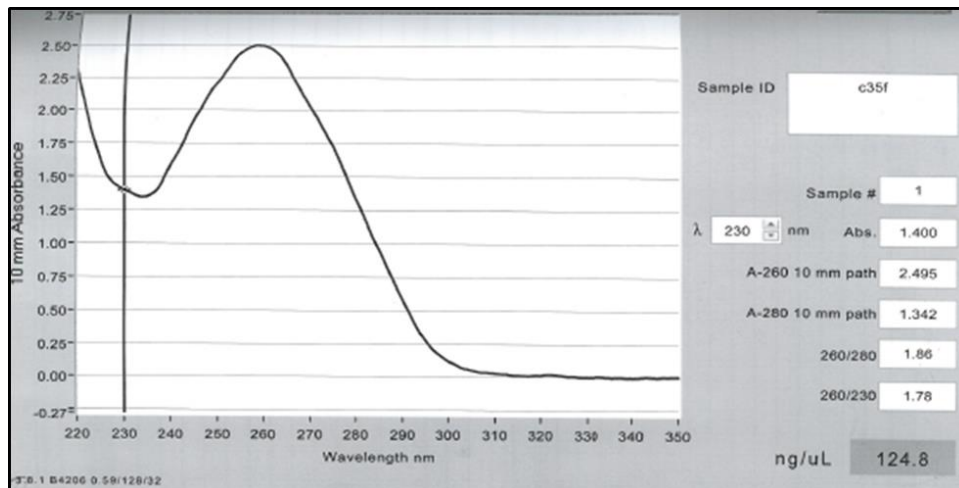


Figure 2: Representation of the spectrophotometric quantitation of a control DNA sample (C35F) before being utilized in the optimisation of the TaqMan™ Genotyping Assay using the NanoDrop ND-1000 (Nanodrop Technologies, USA)

