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The application of the N-terminal fragment of pro hormone brain natriuretic Peptide (NT proBNP) in cardiac disease risk assessment.

BY

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Thank you God for such a challenging opportunity, without these tough hurdles we cannot grow and for fill our earthly purpose

Dedication

I dedicate this work to my parents who have always believed in me, as well as my husband and son who make every task worth doing.

Summary

Candidate: Yvette Hlophe

Title: The application of the N-terminal fragment of the pro-hormone brain natriuretic peptide (NT proBNP) in cardiac disease risk assessment

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NT proBNP is a precursor peptide that is derived from proBNP a cardiac natriuretic peptide (NP), which is measured in plasma to assist with management of cardiac related conditions. The first aim of this study was to examine the biological variation (BV) of NT proBNP and to calculate its reference change value (RCV) in healthy individuals. The second aim was to assess if the RCV calculated for NT proBNP could be used as prognostic marker in patients who presented with chest pain or acute coronary syndrome (ACS) symptoms at an emergency care unit. A second NT proBNP measurement was performed twenty four hours after admission. These patients were assessed for a likelihood of presenting with three outcomes; probability of mortality; angioplasty or bypass surgery after being monitored for a period of one year. The third aim of the study was to investigate if NT proBNP could be used to identify individuals with possible cardiac conditions for life insurance purpose.

NT proBNP assays were measured using the Elecsys 2010 analyser from Roche Diagnostics which uses an electrochemilumeinesence immunoassay that functions on the basis of the sandwich technique. Statistical methods will include analysis of variance and Odd Ratio analysis to assess the risk of a particular outcome.

The reference change value (RCV) was calculated as 67%. The odds of requiring angioplasty increased 3.6-fold if a RCV of >67% was observed. The RCV did not aid in predicting mortality, or in predicting need for bypass surgery. A significant association was observed between ECG findings and NT proBNP. The ECG and NT proBNP are not independent of each other in males, as is evident from the correlation between the two variables ($r=0.6$; $p<0.005$). This implies that the variables can be used interchangeably.

The study concluded that the 67% RCV calculated allows for a more accurate prognostic analysis of each patient and it allows us to move away from the use of population based reference ranges. It was established that a 67% RCV can significantly indicate the likelihood of patients requiring angioplasty. The correlation that was assessed between NT proBNP and the ECG provides insurance companies with the option to choose the method that best accommodates them when assessing cardiac risk.

Keywords: NT proBNP, natriuretic peptides, proBNP, biological variation (BV), reference change values (RCV), electrocardiogram (ECG), bypass surgery, mortality, angioplasty, Odd ratios

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NP natriuretic peptides

BV biological variation

NT proBNP$_{1-76}$ N-terminal fragment of the pro-hormone brain natriuretic peptide

RCV reference change value

ACS acute coronary syndrome

ECG electrocardiography

ANP atrial natriuretic peptide

BNP brain natriuretic peptide

BNP-32 one of the precursor molecules of the brain natriuretic peptide

Pro BNP polypeptide molecule of brain natriuretic peptide

LV left ventricular

cDNA complementary deoxyribonucleic acid

NPR-A atrial natriuretic peptide receptor- type A

cGMP cyclic guanosine monophosphate

NPR-C atrial natriuretic peptide receptor- type-C (clearance receptor)

NEP neutral endopeptidase

COOH carboxylic acid

MI myocardial infarction

LDL low density lipoprotein

ROS oxygen free radicals

NSTEMI non-ST segment elevation myocardial infarction

STEMI ST segment elevation myocardial infarction

NSTEACS non-ST elevation acute coronary syndrome

UA unstable angina

AMI acute myocardial infarction

TnI troponin I

TnT troponin T

TnC troponin C

CK-MB creatine kinase consists of two subunits brain type [B] or muscle type [M]

SEP socioeconomic position

SST serum separating tube

SD standard deviation

CV coefficient of variance

BMI body mass index

CV_i within individual coefficient of variance

CV_a analytical coeffient of variance

CV_g between coeffient of variance

TE_a analytical total error

Pg/ml pica grams/ millilitre

Rpm revolutions per minute

Cal set calibrator

URL upper reference limit

LRL lower reference limit

ROC receiver operating characteristic

Chapter 1

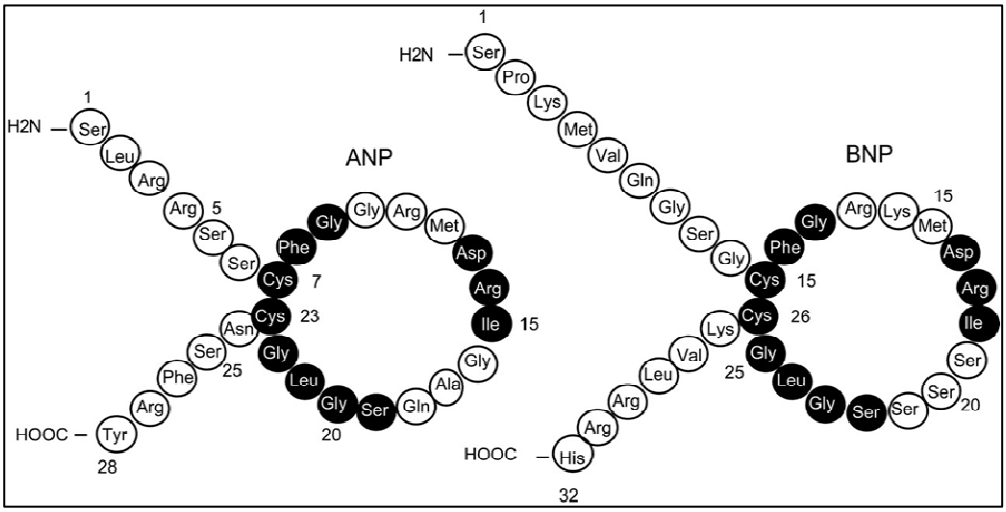
Introduction and literature review

1.1 Physiology and biochemistry of NT proBNP

1.1.1 Background

The notion of the heart participating in activities that were endocrine related came about more than five decades ago (1). Microscopic visuals assisted in recognising intracellular granules commonly found in endocrine cells in atrial myocyte cells (2). The full endocrine role of the heart was confirmed by several researchers: deBold and his colleagues injected derivatives from atrial myocytes in rats and they observed quick depictions of natriuresis and diuresis (3). Flynn demonstrated that the natriuresis was brought about by a peptide found in the atrium of the heart known as atrial natriuretic peptide (ANP) which structurally is represented by 28 amino acids and a disulphide bond(4). In 1988 research conducted by Sudoh demonstrated a second natriuretic peptide they found in porcine brain called brain natriuretic peptide (BNP) (5). Further studies established that BNP is also produced in cardiac myocytes and that these two cardiac natriuretic peptides were also found to share common receptors (6).

Figure 1.1 Cardiac natriuretic peptides



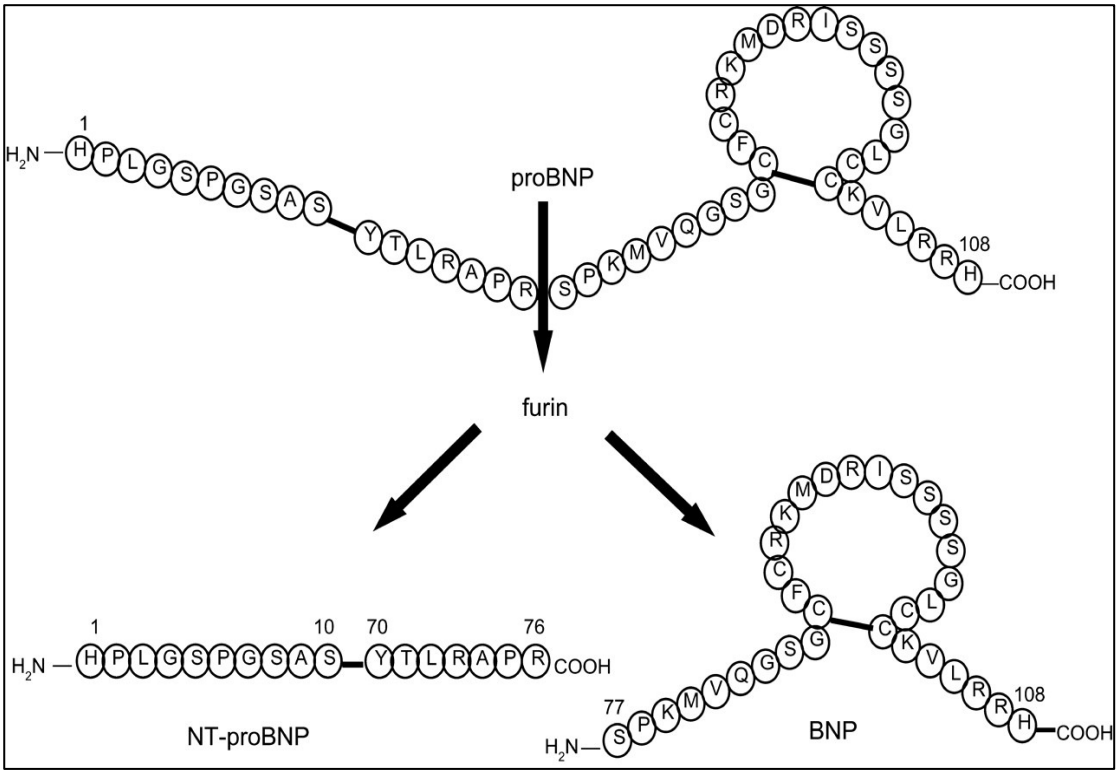
Legend: Cardiac natriuretic peptides ANP (28 amino acids) and BNP (32 amino acids) are homologous in structure, forming a ring with a disulphide bridge.

Source: Hall C Eur J Heart Fail 2004; 6:257-260

1.1.2 Structure of NT proBNP

The polypeptide proBNP is a molecule in humans that encodes 108 amino acid residues. The first chromosome is where the BNP gene is found (7). After cleavage of the proBNP molecule by the protease (furin or corin) the N-terminal fragment NT proBNP (1-76 amino acids) is separated from the second region BNP-32 at the C-terminal (77-108 amino acids). It is named BNP-32 because of the size of the molecule and its biological effects (8). The ring structure at the C-terminal is common in other natriuretic peptides and it is formed by the disulphide bonds at location 86 and 102 by the cysteinyl residues. The disulphide bond is important when it comes to receptor binding and physiological action (9).

Figure 1.2 Schematic drawing of proBNP and its derivatives



Legend: Schematic drawing of proBNP showing enzymatic cleavage into biologically active BNP and NT-proBNP

Source: Hall C Eur J Heart Fail 2004; 6:257-260 (7).

1.1.3 Storage and release of NT proBNP

In the cardiac myocytes the atrium and ventricles can both express the BNP gene. In a non-diseased heart the BNP gene is expressed prominently in the atrium (10, 11). In a diseased heart BNP gene expression is up-regulated in the ventricles (12). The atrium and ventricle myocytes may both express the BNP gene but their intracellular storage and secretion methods differ. Secretary granules are found in the atrium that store the intact form of the peptide proBNP as well as the precursor molecules, NT proBNP and BNP-32 (1-2). In a non-diseased heart the secretory granules are not detected in the ventricles and neither are the precursor molecules of proBNP (11, 13). Although reports have been released that state that within the ventricles of a diseased heart the secretory granules and precursor molecules have been detected (13, 15).

It seems that storage of the precursor molecule NT proBNP in secretory granules is done in small quantities; it is the demand of the peptide that leads to up-regulation of the BNP gene in the ventricles. The up-regulation of the ANP gene is a less timely action (16).

When there is stress on the walls of the cardiac myocytes and one looks at atrial activity there is a higher production of BNP in relation to ANP, even after a myocardial infarction up-regulation of the BNP gene exceeds the up-regulation of the ANP gene (17).

It seems that other cells of the heart also express the BNP gene, cardiac fibroblasts (18), as well as coronary vasculatures in coronary atherosclerosis (19). A study by Casco VH *et al* also showed that atherosclerotic plaques are able to produce and release natriuretic peptides, this was discovered when natriuretic peptides and their receptors were identified within the atherosclerotic plaques of the coronary arteries (20). Indicating that NT proBNP levels are linear to the size of the atherosclerotic plaque in the absence of LV systolic dysfunction (20, 21) The quantity of NT proBNP

from the atherosclerotic plaque that contributes to the NT proBNP in the circulating system is unknown (22).

BNP (B- natriuretic peptide) is released by intact cells, meaning cells that are not undergoing ischemic activity, meaning the pathophysiological consequences of ischaemia can be determined or measured instead of the amount of myocardial damage or infarction that is usually established. This means that BNP can facilitate in the measurement of myocardial ischaemia as an individual parameter that would not be detected by markers of myocyte necrosis (23).

The distribution of the NT proBNP quantity over the large spectrum of ACS is associated stringently to the prognosis of the patient (24-26, 27-30). NT proBNP levels are affected mainly by left ventricular (LV) wall stress, ischemic states, and the presence of atherosclerotic plaques (22). The long term belief that NT proBNP levels where elevated due to left ventricular diastolic or systolic dysfunction propagated by myocardial ischaemia resulting in increased wall tensions. Recently studies show that cardiac myocytes can release NT proBNP as feedback to the myocardial ischaemia without the presence of ventricular wall stress (31, 32). NT proBNP levels seem to even be probed by short term ischaemia induced by angioplasty activity (33).

1.1.4 Physiological processes of NT proBNP

Previously it has been suggested that proBNP was cleaved by furin a ubiquitous endoprotease, because the genes for both furin and BNP are expressed in cardiac cells of a diseased heart (34, 35). As research evolved a serine protease called corin was sighted on the cDNA of the human heart (36, 37). This serine protease cleaves proBNP and proANP *in vitro* and it has been assumed that it does this at a similar cleavage site (38, 39). The corin cell membrane contains a trans-membrane domain suggested of cleaving the precursors (BNP-32, NT proBNP) once it is secreted (39), although it is not known exactly where corin cleaves the polypeptide proBNP (40).

Physiological *in vitro* studies on the B- natriuretic peptide (BNP) indicate that it binds to the natriuretic peptide receptor A (NPR-A) on the surface of the target cells and results in the stimulation of the second messenger cyclic guanosine monophosphate (GMP) which activates the biological effects of the natriuretic peptides (41). These biological effects include diuresis, vasodilatation, and inhibition of renin and aldosterone production as well as cardiac and vascular myocyte growth (7). In addition BNP binds another receptor NPR-C known as a clearing receptor. BNP has a longer plasma half-life than ANP because of the lower affinity of NPR-C for BNP in human beings (41.)

It is not known if NT proBNP has its individual biological effects or if the polypeptide proBNP can bind to peripheral receptors (7.)

1.1.5 Clearance of NT proBNP

The natriuretic peptides are cleared from plasma by binding natriuretic clearance receptors (NPR-C), but there is a secondary clearance mechanism by neutral endopeptidase (NEP). The longer half-life of BNP when correlated to ANP is also attributed to its seemingly resistant response to NEP (42).

1.1.6 Measurement of proBNP derivatives in plasma

Most proBNP is cleaved before it reaches plasma circulation (43), so its derivatives circulate easily in plasma. This occurs in response to wall stress experienced by the ventricles and atrium. The molecular diversity has been assessed using chromatography and immunoassay techniques, because of the diversity found in the breakdown of the precursor molecules (BNP-32, NT proBNP) there is no accuracy in plasma levels, especially when they are measured by different assays using different epitope- specific antibodies, (44) that is why a standardised assay was developed from Roche diagnostics for better accuracy. The immunoassay technique uses antibodies elevated against the COOH terminus of NT proBNP 1-76 in plasma.

1.1.7 Comparison of the precursor molecules BNP-32 and NT proBNP

In comparison to BNP-32 the longer half- life of NT proBNP does not make it as vulnerable to the individual variations [pre-analytical, analytical, and inherent] (45, 46). BNP-32 is more prone to variability, because it is not stable at room temperature and the BNP molecules begin to break down once a blood sample has been collected, before processing (47).

In terms of analytical performance of our analytes studies tend to favour NT proBNP assays over BNP-32 (48). NT proBNP has a half hour assay time, it presents with more sensitivity and efficiency than the BNP-32 assay (49). NT proBNP has agreeable reagent stability and is automated. Assay costs for NT proBNP are 40% lower than the price for a BNP-32 assay. NT proBNP is becoming the amicable choice in clinical practice according to Forseca *et al* and Fuat *et al* (50, 51).

A number of comparative studies where done that suggested that NT proBNP may be a more positive marker than BNP-32 for asymptomatic structural heart disease. Hunt *et al* (52) indicated the sensitivity of NT proBNP over BNP-32 in the detection of LV dysfunction due to the presenting longer half-life. This was backed by Seino *et al* (53) and Fonseca *et al* (51) they also added that NT proBNP was not only more sensitive but also more specific to LV dysfunction. Vanderheyden *et al* suggested that the more stable structure of NT proBNP contributed to the high sensitivity and specificity that NT proBNP showed for LV systolic dysfunction (54) and Mackie *et al* supported this (55). Although there is physiological evidence to support the sensitivity of NT proBNP as a marker for LV systolic dysfunction it is yet to be substantiated in a study with a large population (56). There are authors who disagree with the beneficial traits of NT proBNP and who claim no difference in the analytical performance of NT proBNP verse BNP-32 (57-59).

Table 1.1 Comparison of the precursor molecules BNP and NT proBNP

	BNP	NT proBNP
Half-life	20 minutes	60-120 minutes
Regulation	Transcription	Transcription
Pre-analytic	Minimal	Minimal
Stability	24 hour EDTA at 2-8 degrees Celsius	72 hour EDTA at 2-8 degrees Celsius

Legend: This table compares the half-lives of the natriuretic peptides it also looks at the temperatures and mediums in which they are most stable. It also shows how the peptides can be affected by pre-analytical error and their regulation mechanisms (41).
Source: Ind J Clin. Biochem 2007; 22(1):5 (41).

1.1.8 NT proBNP as a prognostic indicator in acute coronary syndrome patients

There is ample data that supports the use of the B-natriuretic peptide value for heart failure, but more recently there is interest in the use of B-natriuretic peptide for prognostic use in Acute Coronary Syndromes (ACS). Encompassing the full spectrum of ACS, from those without myo-necrosis unstable angina (UA) to those with irreversible myocyte injury myocardial infarction (MI). Ventricular wall tension increases either due to an ischemic event or as a result of infarction and natriuretic peptides provide the present information on the pathophysiology and outcomes of the ACS's (23).

A large number of studies have reported the use of the B-type natriuretic peptide in patients presenting with the ACS as a prognostic marker (60, 61). Studies either measured BNP-32 or NT proBNP within the sub-acute phase after admission and they all indicated the precursor peptides to be good indicators of mortality (62).

1.1.9 Biological variation of NT proBNP in ACS patients

This study focused on the biological variation of NT proBNP as a starting point because it is important to ensure that the analyte is interpreted correctly, each result presents with its own inherent random variation. This random variation comprises of all the variations the pre-analytical and analytical variations from laboratory workings as well as the inherent variations (within [inter] and between [intra]) of the analyte. To overcome these variations it was important to calculate a reference change value (RCV) which is actually the vital difference that must be surpassed between two sequential test results for a meaningful change to occur. Once a RCV is calculated and the individuality of an analyte is understood, analysis of the test results is more accurate and therefore provides more reliable prognostic information.

There are intra-individual changes present within NT proBNP. Studies have shown that for NT proBNP readings to be clinically significant there should be at least a change of 50-66% from one week to another (63). The intra-individual variations that are detected from NT proBNP are indicative of biological variations that can probably not be recognised by the present clinical analysis techniques (56).

1.2 The Acute coronary syndrome (ACS)

1.2.1 Causes of the acute coronary syndrome

There are five principal causes of ACS:1 Plaque rupture with acute thrombus; 2 progressive mechanical obstruction; 3 inflammation; 4 secondary unstable angina, because of severe anaemia or hyperthyroidism; and 5 dynamic obstruction, which may be due to a focal spasm of an epicardial artery. It is not common that a patient with ACS will present with a single cause, it is more common that a patient presents with a combination of these causes. Therapeutic approach has to be unique for each individual depending on the severity of the causes that arise (64).

1.2.2 Pathophysiology

Atheromatous plaque disturbances are cardinal to the development of the acute coronary syndrome. Atherosclerosis is caused by low density lipoprotein (LDL) that has been exposed to free radicals, especially oxygen free radicals (ROS), when these oxidised LDL molecules come into contact with arterial walls they cause some damage. The body responds by releasing macrophage white blood cells to the site of injury but these white blood cells are unable to tolerate the oxidised LDL and these white blood cells become loaded with fat cells that the LDL cells are actually responsible for carrying. The loaded white cells eventually rupture and they deposit a great amount of the oxidised cholesterol in the artery wall, each plaque rupture is accompanied by a thrombus (clot) to cover the plaque rupture, eventually this triggers an inflammatory response in the arteries. The cholesterol plaques that have formed cause the arterial muscle cells to expand and a hard cover is formed over the site of injury, the hard cover that forms minimises blood flow in the affected artery and increases the blood pressure. The reduced blood flow in the arterial walls also known as an ischaemic condition is what results into the acute coronary syndromes (65).

Non-ST segment elevation myocardial infarction (NSTEMI)

The blood clot or the thrombus in these patients is semi obstructive, leading to ischaemia without persistent ST-segment elevation (64). The period of ischaemia as well as the severity and the amount of myocardial damage caused is what distinguishes NSTEMI from unstable angina (64).

ST segment elevation myocardial infarction (STEMI)

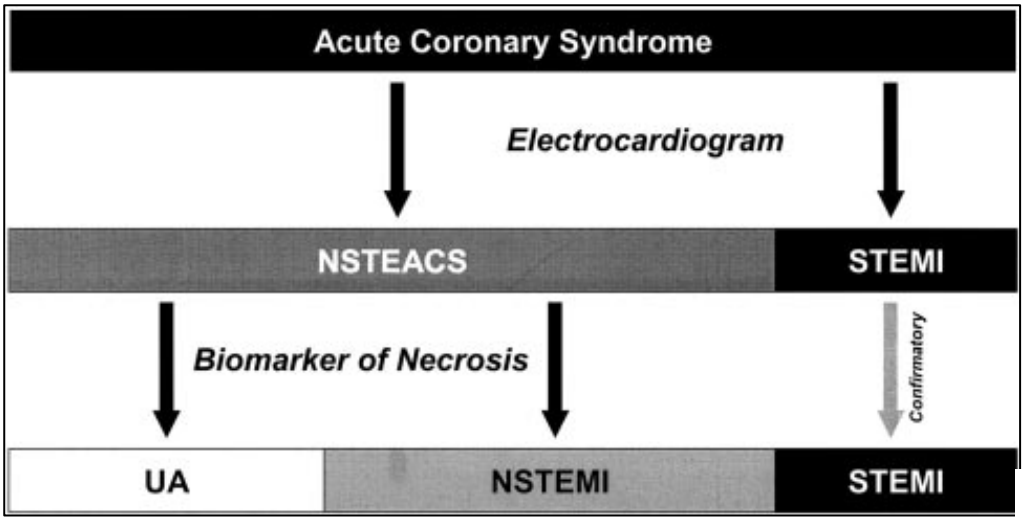
The intra-coronary clot completely blocks the blood vessel in question, resulting in STEMI (64).

1.2.3 Categorisation

ACS is progressive in its severity; it starts of as asymptomatic, develops through stable and unstable angina, progresses into non-Q wave MI, then ends in trans mural MI, cardiac arrhythmia, and finally ends in death. The end stage of the ACS is myocardial infarction which is initiated by acute myocardial ischaemia (66). Acute myocardial ischaemia causes chest pain due to insufficient blood supply to the heart muscle that results from coronary artery disease (also called coronary heart disease).

On arrival to casualty unit patients with ACS are categorised in two major groups based on their electrocardiogram (ECG) prediction. Patients with new ST segment elevation on the ECG are diagnosed with acute ST segment myocardial infarction (STEMI) and those who present with ST segment depression, T-wave changes, or no ECG abnormalities are categorised as (non-ST elevation ACS, NSTEACS). The latter term (NSTEACS) incorporates unstable angina and non-ST elevation myocardial infarction (NSTEMI). Unstable angina and NSTEMI are closely related conditions, having in common, pathogenesis and clinical presentation but their intensity varies (64).

Figure 1.3Categorisation of acute coronary syndromes



(64)

Legend: NSTEACS (non-ST elevation acute coronary syndrome) STEMI (ST elevation myocardial infarction) UA (unstable angina) NSTEMI (non –ST elevation myocardial infarction) (64).

Source: European Society of Cardiology/ American College of Cardiology Committee for the redefinition of myocardial infarction. (J Coll Cardiol 2000; 36:959-969)

1.2.4 Signs and symptoms

Ischaemia is not always symptomatic, but that does not reduce the damage to the heart. Symptoms that are experienced are chest pain that spreads from the chest towards the arms, neck, shoulders, or jaw. They could feel immense pressure on their chest, shortness of breath, heart palpitations, and fainting, sweating, and even nausea. Women tend to feel pain high in the abdomen or the back (65).

Unstable angina (UA)

Unstable angina presents with ischemic discomfort, with at least one of three features:

- It occurs at rest (or with minimal exertion), usually lasting >10 min;
- It is severe and of new onset (i.e., within the prior 4–6 weeks); and/or
- It occurs with a crescendo pattern (i.e., distinctly more severe, prolonged, or frequent than previously).

UA is a serious indicator of a pending MI and occurs un-expectantly at rest (67).

1.2.5 Diagnosis

Differentiating Acute Coronary Syndrome from the non-cardiac chest pain is the primary diagnostic challenge. The initial assessment requires a focused history, a physical examination and an electrocardiogram (ECG) reading and serum cardiac marker determinants.

1.2.6 Electrocardiogram (ECG)

The ECG readings provide beneficial information which allows the risk of a patient acquiring ACS to be assessed. It is also beneficial for diagnostic and therapeutic purposes. The sensitivity of the ECG is increased when used for patients with re-occurring chest pain (68). In the fourth chapter of this study we compare the ECG interpretations made by an underwriter versus the analysis of the NT proBNP analyte. The ECG diagram gives an indication of the parameters that have to be considered by the underwriter to determine the severity of the ACS, be it an onset of very current angina, unstable angina, or myocardial infarction: and also the possible presence cardiac hypertrophy associated with cardiac failure. The correct categorisation is crucial as this informs the underwriter the degree of risk the patient provides for the insurance company. Tabulated below in table 1.2 are the ECG findings used to diagnose ACS.

Table 1.2 ECG findings for the diagnosis of acute coronary syndrome

	Lesion	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
ST-segment elevation greater in lead III than in lead II plus ST-segment depression of > 1 mm in lead I, lead aVL, or both	Right coronary artery	90	71	94	70
Absence of the above findings plus ST-segment elevation in leads I, aVL, V_5, and V_6 and ST-segment depression in leads V_1, V_2, and V_3	Left circumflex coronary artery	83	96	91	93
ST-segment elevation in leads V_1, V_2, and V_3 plus any of the features below:					
ST-segment elevation of > 2.5 mm in lead V_1, right bundle branch block with Q wave, or both	Proximal LAD coronary artery	12	100	100	61
ST-segment depression of > 1 mm in leads II, III, and aVF	Proximal LAD coronary artery	34	98	93	68
ST-segment depression of <= 1 mm or ST-segment elevation in leads II, III, and aVF	Distal LAD coronary artery	66	73	78	62

Legend: The table indicates the likely ECG interpretation that will be obtained if a particular coronary complication presents. It also indicates the sensitivity, specificity, positive and negative predicative values that will present with the interpretation.

Source: Zimetbaum PJ, Josephson ME. Use of the electrocardiogram in acute myocardial infarction. N Engl J Med 2003; 348:934; 935 (68)

1.2.7 Biomarkers currently used to diagnose ACS

1.2.7.1 Troponins

Troponins consist of three proteins that are vital in muscle contraction of skeletal and cardiac muscle but not of smooth muscle. Troponin is attached to tropomysin and tropomysin blocks muscle contraction by inhibiting the binding site of the myosin cross-bridge. Muscle contraction occurs when calcium is deposited into the sarcoplasm, by an action potential which stimulates the opening of the calcium channels into the sarcoplasmic reticulum (70).

The type of muscle determines the troponin set up. The TnC subunit of troponin in cardiac muscle has three calcium binding sites, whereas there are four binding sites in skeletal muscle (70). Cardiac myocytes have specific isoforms of troponins that are specific to myocytes, TnI and TnT; these isoforms can be quantified using monoclonal antibodies specific to cardiac epitopes (64). Human troponin I has one isoform specific to cardiac myocytes with a molecular weight 2387 Da and 209 amino acid residues. The troponin I molecule consists of two serines in the 22 and 23 positions. Protein kinase is used to phosphorylate these two amino acid residues in vivo, resulting in four protein forms; one dephospho, two monophospho, and one biphospho exist together within the same cell. Conformation of troponin I is changed by phosphorylation and its relationship with other troponins and with anti- troponin I antibodies is modified. The troponin I within the blood stream is phosphorylated (69).

A minimum of four troponin T isoforms are present in human cardiac myocytes and are presented in a progressively controlled manner. Calcium sensitivity and inhibition is essential to determine if the different isoforms N-terminals are specific with regards to performance and structure. The four isoforms for troponin T are TnT1 (all exons present), TnT2 (missing exon 4), TnT3 (missing exon 5) and TnT4 (missing exons 4 and 5). The N-terminal of troponin T plays a role in calcium sensitivity and this has a

significant influence on the charge generated. The greater the charge the higher the calcium sensitivity and this is primarily due to exon 5 (71).

When considering sensitivity and specificity there is no real clinical difference between the current generations of TnT and TnI. That being said there is actually no standardised assay for manufacturers to use for troponin I material, because of all the different epitopes recognized by the different antibodies used, assay concentration tend to fail. Standardisation for Troponin I will never be possible because of the different antibodies that will always be present in the different assays. Roche diagnostics, a manufacturer has standardised one assay for troponin T (72). Cardiac troponins when compared to other biomarkers of necrosis are associated with fewer false-positive results in skeletal muscle injuries, for example it presents in very high concentrations out of normal range for myocardial injury when the concentration of CK-MB (creatine kinase [CK]: CK enzymes consists of two subunits brain type [B] or muscle type [M] making different isoenzymes of which CK-MB belong) is normal or minimally increased (64).

There has been a trend of patients presenting with elevated troponins, recurring cardiac events and normal serum CK-MB readings, and these patients where suspected of ACS which brought about the relevant need to measure the circulating troponin concentration in patients that were previously classified as unstable angina (64). In the study conducted by Apple FS in 2007 30% of patients with chest pain that would have been diagnosed with unstable angina, without ST-segment elevation, due to insufficient CK-MB elevation have NSTEMI, when observed with cardiac specific troponin assays (72). Unchangeable minor myocardial injury detected by TnT/I may categorise UA patients as high risk for progression to AMI (65). A study done by Marrow and Cannon approximated that 40-60% of patients with definite ACS have baseline troponin concentrations below the clinical decision limit for the assay. Some patients are evaluated directly after an acute MI, for which TnT /I are not yet measurable in serum concentrations, the rest of the patients present with acute myocardial ischaemia without necrosis (unstable angina). Differentiating these two

groups of patients from others with chest pain syndrome that originates from other areas other than coronary ischaemia is a serious clinical dilemma (64).

A recent American Heart Association guideline shows that management of patients with UA and NSTEMI requires an initial sample on arrival and a repeat sample 8-12 hours after symptom onset. Patients without an elevation of the biomarkers after the 8-12 hour period can be side-lined for AMI (66). If a patient presents with TnT<0.06ng\ml or TnT<0.03ng/ml on two specimens>six hours apart this resembles unstable angina. TnI>0.06ng/ml or TnI>0.03ng/ml, these depictions put patients at high risk of ACS (AMI) or depending on the clinical ischaemia, non-ischaemic myocardial damage. TnT>0.5ng/ml or TnT>0.1ng/ml resemble a typical AMI (66).

In a study conducted by Weber *et al*, they indicated that patients with normal range troponin T readings but with high NT proBNP readings indicated either equivalent or a higher probability of mortality than patients with elevated TnT (73).

1.2.7.2 CK-MB

There are three cystolic isoenzymes (CK-3, CK-2, and CK-1) with one mitochondrial isoenzyme (CK-Mt) of creatine kinase (CK) and all of them have been recognised and are differentiated from each other by electrophoresis. Three individual genes have been pin pointed that encode for and are specific for CK-M, CK-MB and CK-BB mitochondrial CK subunits. Even though, CK-3(CK-MM) is highly prominent in both heart and skeletal muscle, CK-2(CK-MB) is more specific for the myocardium, which consists of 10-20% of its total CK activity as CK-MB, in comparison to its reduced quantity of 2-5% in skeletal muscle, and CK-BB found predominantly in the brain (72).

The bands of CK-MM and CK-MB can be separated further at a very high voltage using electrophoresis. A minimum of three isoforms for CK-MM and at least for CK-

MB (subtypes of individual isoenzymes exist. CK-MM3 is the tissue isoform of CK-MM. A time-dependant carboxypeptidase hydrolysis of the C-terminal lysine residues occurs, when CK-MM3 is released into circulation, producing at least two post-translational products: CK-MB2 and CK-MM1 (72).

A B chain negative product and a product that does not contain lysine on both chains are produced once the CK-MB tissue isoform enters the circulation and stimulates carboxypeptidase cleavage of the CK-B carboxy-terminal lysine residue. Electrophoresis is used to separate the two forms of which minute amounts are produced, the M-chain negative and the B-chain positive form, these are the only forms that can be used diagnostically and are labelled CK-MB2 and CK-MB1. The rate of clearance of the total CK activity in blood is dependent on the rate of clearance of all the isoforms. The longer half- lives are associated with the post-translational degradation isoforms. The order of half-lives are CK-MM1>CK-MM2>CK-MM3 and then CK-MB1>CK-MB2 (72). Regular skeletal muscle depending on position contains very little CK-MB, on average < 2% is the common analysis. There are a few differences when it comes to race and slow versus fast twist muscle. Skeletal injuries after casualty can lead to increased absolute elevations of CK-MB above the reference limit of CK-MB in serum. This being said, the percentage of CK-MB would have a low detection because presented percentages change when looking at some activities verse others, a percentage <5% is used to relate CK activity with CK-MB mass, but the percentage preferred is < than 2.5%. A consistent increase in CK-MB due to chronic muscle disease usually appears in patients with muscle dystrophy, end stage renal disease, or polymyositis as well as in healthy patients who participate in extreme exercise and therefore elevate their CK-MB tissue concentration. During the production of skeletal muscle CK-MB genes are activated, that are very alike to those found in the heart, which results in the increased CK-MB levels in skeletal tissue. The CK isoenzyme composition in skeletal muscle can become like that in the heart muscle that is why patients with polymyositis can present with up to 50% CK-MB elevation (72). Assays for CK-MB mass offer superior analytical and diagnostic performance over the assays of CK-MB activity. CK-MB mass is essential in assessing more recent MI or to confirm re-infarctions (66).

1.2.7.3 Myoglobin

Myoglobin is a heme-protein, with a low molecular weight with good positioning in the cytoplasm which results in its early appearance into the circulation after skeletal and cardiac injury. The myoglobin protein in skeletal and cardiac muscle is identical, so injury to either tissue will result in increased serum concentrations, but it is off-course not specific to a particular tissue (72).

Myoglobin is usually requested as a test to be run in correlation with CK-MB and the troponins; it is unfortunately not very specific to MI but is a very sensitive marker (66).

1.3 Study objectives

There are various questions that this project would like to answer about the ability of NT proBNP analyte:

- Can Serum NT proBNP be used as prognostic marker in ACS patients when reference change values (RCV) are considered?
- Can NT proBNP serum levels be used for screening purposes to evaluate ACS risk for life insurance purposes?

To answer these questions a thorough understanding of the biological variation of NT proBNP is required. Therefore, this study should be able

1. To determine indices of variation of NT proBNP measurement;

2. To evaluate NT proBNP as screening test for ACS risk; and

3. To investigate the relationship between NT proBNP and risk factors for ACS.

1.4 Significance of the study

1.4.1 General introduction on the significance of the study

Cardiovascular disease is of great concern in our modern world as it has contributed greatly to increased mortality rates and has left a large percentage of the population impaired world-wide. The management of the contributors such as (smoking, hypertension, and hypercholesterolaemia) in a timely fashion plays a crucial role in the progression of these contributors into a cardiovascular disease. The efficiency of health care providers also plays a crucial role. The funding of further research in this field will also probe further to get solutions to confront this problem (74).

The determining factor for prognosis in the past was the presence or absence of cardiovascular risk factors and this technique was applied internationally. This was a real problem because there was no true relationship with the severity of patient's condition and the treatment they received. Hence the need for a multifactorial cardiovascular risk assessment that allows high- risk individuals to be identified and treated based on the cardiovascular risk of each patient is essential (74).

1.4.2 Social and economic factors related to cardiovascular complications

It was suggested in this study conducted by Agabiti N *et al* that there was a clear relationship between a person's socioeconomic position (SEP) and outcome after cardiac surgery. In this study the difference in mortality rates in private and public hospitals is related firstly to the better pre-operative status of the patients in private hospitals. Secondly those patients in public hospitals wait longer for their surgeries which could explain their bad pre-operative status and therefore increasing the number of bad surgery outcomes (75).

A report conducted by the medical research council indicates that the cardiovascular disease burden is high in South Africa (76). After infectious causes, hypertension ranked second as a cause of high mortality rates in South Africa. In a study done by the internal Medicine Department, School of Medicine at the University of Pretoria,

death rates from obesity, high cholesterol levels and diabetes were respectively ranked fifth, seventh and eighth in importance. Risk factors associated with coronary artery disease for example hypertension, diabetes and the metabolic syndrome where prominent within the South African black population (74).

In summary the socioeconomic disadvantage is associated with higher prevalence of cardiovascular risk factors, morbidity, and mortality from cardiovascular disease, reduced access to specialist care as well as appropriate treatment.

1.5 Methodology

1.5.1 Determination of the biological variation of NT proBNP

Source: healthy volunteers from the chemical pathology department at the University of Pretoria. They all voluntarily signed consent forms to participate in this study and they agreed to be available weekly for sample collection.

Population: 14 healthy individuals as approved by the bio-statistician. Inclusion criteria: wide age range the study group ranged from 26-60 years of age, all races, and both genders. Exclusion criteria: any history of heart complications and factors related to heart complications for example (elevated blood pressure, obesity, elevated cholesterol).

Sample: 1x SST tube (contains a gel separator and a clot activator) of blood was drawn, first thing in the morning. A sample was collected once a week for five consecutive weeks per volunteer.

Measuring instrument: each sample per patient for each week was run in duplicate using the Elecsys 2010 Immunoanalyser.

Data collection: results were tabulated in an excel spread sheet, each sample, per individual, per week that was run in duplicate.

Data analysis: the results were used to calculate the mean, standard deviation (SD) and coefficient of variance (CV) for the analytical, pre-analytical and inherent

(inter/intra) variations. The main parameters calculated were reference change value (RCV) and index of individuality (II) of NT proBNP.

1.5.2 Use of reference change values for NT proBNP as a prognostic marker in acute coronary syndrome

Source: casuality patients from Unitas or Heart hospital presenting with ACS symptoms and who were admitted. Consent was obtained from both the casualty unit superiors the Cardiologists and the individual patients.

Population: 109 participating patients as approved by the bio-statistician. Inclusion criteria: >21 years of age, genders and all races, chest pain or ACS symptoms. Exclusion criteria: abnormal renal function, poor ejection fraction <45%, serum troponin levels less 0.03mg/ml.

Sample: 1xSST serum separating tube of blood was drawn on admission at the casualty unit and again 24 hours following admission on each patient. The samples were centrifuged, aliquoted and stored at – 80 degrees Celsius until all samples were collected.

Measuring instrument: all samples were thawed on the same day, each sample analysed in duplicate for NT proBNP using the immunoanalyser the Elecsys 2010.

Data collection: after a period of one year of each patients initial casualty admission their doctor was contacted to establish if the patient had received angioplasty or bypass surgery or if they had died. These are the study outcomes we focused on.

The samples analysed by the Elecsys 2010, per sample in duplicate were tabulated on an excel spread sheet.

Data analysis: logistic regression analysis was used to assess correlation with our study outcomes (angioplasty, bypass surgery, and mortality) with our tabulated NT proBNP readings considering the RCV calculated for the analyte from our healthy population.

1.5.3 Use of NT proBNP to identify individuals with possible cardiac conditions for life insurance purpose.

Source: patient data was obtained from Momentum health, on applicants applying for life insurance

Population: 106 individuals were studies, for each applicant data was provided on their (age, gender, BMI, smoking status, cholesterol levels, systolic and diastolic values, as well as their ECG readings). A sample of aliquoted serum on each patient was provided for NT proBNP analysis. The aliquoted samples were stored at -80 degrees Celsius, thawed on the same day and run in duplicate on the analyser.

Sample: the aliquoted serum sample provided for each insurance applicant was run in duplicate on the analyser.

Measuring instrument: the immunoassay analyser the Elecsys 2010 was used once again to analyse the samples for NT proBNP.

Data collection: tabulated on an excel spread sheet is the sample reading that was run in duplicate for each sample with all the other data provided per individual (age, gender, BMI etc.)

Data analysis: regression analysis was used to assess any correlation with elevated NT proBNP and all the other data provided for each individual, especially elevated NT proBNP with abnormal ECG findings, as well as to assess if age and NT proBNP were independently related to an abnormal ECG finding.

1.6 Summary

The current method that is followed is that a diagnosis of ACS is made based on the patient's history, ECG and the high concentrations of the biochemical markers. The same parameters are used to assess the risk of an adverse outcome (77).

What we know about the analyte is that: NT proBNP analysis is said to be useful in risk assessment when analysed at onset to a casualty unit or for prognostic decision making, (78, 79-84) and that natriuretic peptides are good forecasters of mortality in ACS patients (85).

What this study hopes to establish is to assess the clinical and statistical significance that NT proBNP can add to the diagnoses and more importantly prognosis of ACS patients, this will be achieved by collecting data on the biological variation to be able to interpret the changes in serum NT proBNP levels over time. There is currently insufficient data on the intra individual biological variation (CV_i) of NT proBNP, which limits the understanding of the variations in concentration on disease progression or treatment optimisations (86).

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Chapter 2

Determination of the biological variation of NT proBNP

2.1 Aim

To study the biological variation of NT proBNP

- In order to calculate a reference change value (RCV) for NT proBNP
- To understand the individuality of the analyte by calculating the index of individuality for NT proBNP

2.2 Introduction

2.2.1 Biological variation (BV)

When considering how to interpret serial results of an analyte measured in a laboratory it is important to consider the human biological variation of the analyte. The human biological variation considers the variation between and within the individuals for the analyte being measured (1). By collecting serial blood samples from healthy individuals it is possible to study the BV of NT proBNP. The variation encountered by a particular analyte is categorised based on the origin of the variation.

Pre-analytical variation which refers to all activities that are conducted prior to the sample collection (2). Analytical variation which presents in two forms either random (precision) or systemic (bias).

The method used to analyse a sample and the diagnostic system used, directly impacts the random variation, this could stem from temperature irregularities, poor reagent handling or insufficient sample quantity. A method with good precision has a low random variation, but in a method with poor precision the random variation will impact negatively on the quality of clinical analysis (2).

Systemic variation or bias is known as the deviation from the acquired value to the true value. Bias is not consistent and can vary with changes made to instrumentation and methodology, the differences of samples run before and after the changes made to the instrument or method will add to the variability over time of the analyte (2).

The aim should be to always reduce bias before any analytical reports are realised. If there are various assays for an analyte, they all have their own biases, one assay system should be picked as a gold standard, and all other assay systems should be calibrated to the gold standard as is done with the NT proBNP assay from Roche Diagnostics (2).

If the pre-analytical variation is carefully monitored and the experimental analytical variation is quantifiable, it is plausible to calculate the authentic within and between subject biological variation values. This will be useful to set quality control parameters so it is possible to determine the relevance of serial result modifications in an individual and to still be able to determine the use of reference ranges (2).

The BV is made up of within and between variations, when assembling data from within and between biological variations a smaller population is studied and instead of one sample per person, many samples are taken per individual, in comparison to studies that look at pathology (2). The parameters of biological variation are used to put in place quality specifications for bias, analytical total error ($TE_{a)}$ and precision, to monitor changes in serial results of an analyte and to evaluate the clinical use of population based reference intervals by calculating the analytes index of individuality (3).

Desirable quality specifications for precision, bias and TE_a have been connected to the within BV (CV_i) and the between BV (CV_g) of analytes measured in the laboratory (2, 4-5). An enticing imprecision should be less than one half of the within BV= CV_a<0.5CV_i. A desirable bias is defined by <0.25$[(CV_i)^2 + (CV_g)^2]^{½}$ which means

the bias should be less than a quarter of the group biological variation. The total error (TE) is TE_a< bias+1.65 CV_a (using a 95% probability) P< 0.05. The total variation is measured or denoted as: $CV_T = (CV_a^2 + CV_i^2)^{½}$ (3).

In figure 2.1 the total error concept is explained which compensates for the effect of both the within and between variation of the analyte under analysis. Total error is calculated by adding precision and bias, whether the bias is positive or negative. Work is conducted at 95% probability, to give room for 5% error; the 5% error is removed from the upper and lower levels of the distribution. Actually only 90% is included in the distribution with a multiplier of 1.65 (2).

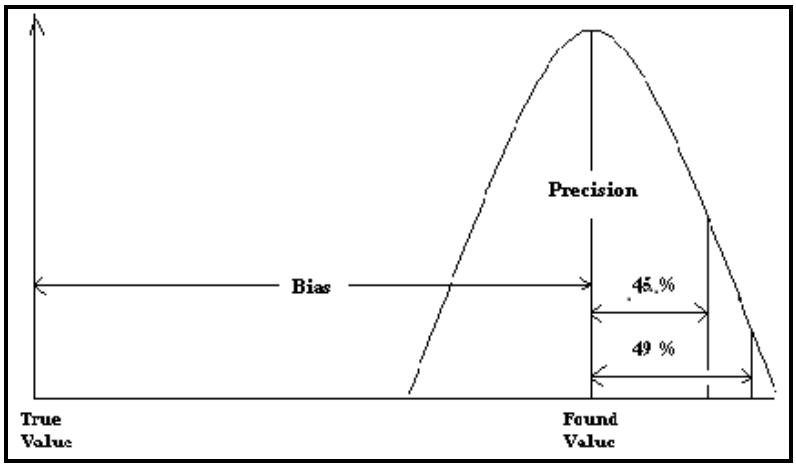
Figure 2.1 The Total Error Concept

The formula for total error allowable then becomes

TE=bias +Z*precision, or

TE=bias+1.65*precision for 95% probability

The basis of this formula is shown in figure 2.1.



Source: Frazer C. Biological Variation: From principles to practise. 2001 (2).

Quality specifications used in laboratories such as precision, bias and total error , all have their pros and cons, but the priority is that the quality specifications meet the demands of accurate clinical decision making (2).

It is clear now that any changes that occur in serial results of an individual after the lapsing of time are due to three factors either: pre-analytical, analytical (precision/bias) or biological (within/between) variation. If the pre-analytical variation is not considered a contributing factor and the biological variation is a constant variable, it only leaves our random and systemic variation as factors. Meaning the change in serial results is due to the analytical precision denoted as: $CV_a < 0.5CV_i$ [meaning the analytical coefficient of variation should be smaller than half of the within-subject biological variations coefficient of variance] (2).

2.2.2 Reference change value

The RCV theory was established by Harris and Yasaka, so they would be able to recognise relevant changes in diseased patients when monitoring them (6, 7). The RCV theory can be applied to serial laboratory results to expose a problem before the clinical manifestations become visible (8). The RCV is calculated using the within CV_i and the analytical CV_a variations when looking at serial laboratory results (8). The contrast between laboratory noise and a prognostic diseased state can be made clearer when the RCV is calculated, which allows for better patient prognosis (9-13).

A change in values when analysing serial results of an analyte is very likely, but whether the change is significant or not really requires the RCV to be calculated and this is highly suggested by Harris and Brown (14).

The RCV is defined as the vital change that must be surpassed between two sequential results for a significant difference to develop (2). $RCV = 2^{½} * Z * (CV_a^2 + CV_i^2)^{½}$
The RCV is the added value of both variations multiplied by a Z score, which is reliant on the probability chosen for statistical significance and the expected direction of change (either increase or decrease in value). For bidirectional change, with a probability of 95% ($p<0.05$) we use a Z score of 1.96 while a highly significant result with a probability of 99% ($p<0.01$) we use a Z score of 2.58 (15).
RCV is also influenced by changes in the bias of a technique. The RCV is flawed in that most data of CV_i is on healthy individuals who may lead to numerous false positives, when applied to a sickly population. Despite this factor it can be used in prognostic analysis (2).

2.2.3 Index of individuality

Biological individuality: an analyte can present with either a low index of individuality (<0.6) or a high index of individuality (>1.4). A low index of individuality means that the analyte has marked individuality. If a person gets sick and their results are affected there will be a variation in the homeostatic set point for that individual but that variation will still lie within the conventional reference intervals. Laboratories would not flag this as unusual because population based reference intervals are used and not RCVs (2).

If an analyte has a high index of individuality this means the analyte has little individuality. If an individual is ill, results are affected and any deviations from the individual's homeostatic set point will flag out of the reference interval and this result will be flagged by the laboratory as unusual. That is why analytes with a high index of individuality are not affected by reference intervals but analytes with a low index of individuality are highly compromised with the use of population based reference intervals (2).

The low index of individuality is determined when the $CV_i << CV_g$ (the coefficient of variations within-subject biological variation is smaller than the between-subject coefficient of variation). With analytes with a low index of individuality, significant changes with the analyte only become visible as the disease progresses drastically and the homeostatic set point for that individual gets closer and eventually surpasses the limits of the reference intervals (2).

2.3 Methodology

2.3.1 Study population group

14 healthy individuals, volunteers from the Chemical Pathology department at the University of Pretoria. The numbers of participants were approved by the bio-statistician. They all voluntarily signed consent forms to participate in this study and they agreed to be available weekly for sample collection. *Inclusion criteria:* healthy

individuals, the study group ranged from 26-60 years of age, all races, and both genders. *Exclusion criteria:* any history of heart complications and factors related to heart complications for example (elevated blood pressure, obesity, elevated cholesterol).

Table 2.1 Description of the study population

INDIVIDAUL	RACE	AGE	SEX
1	White	55	F
2	White	54	F
3	Indian	30	F
4	White	45	F
5	White	37	F
6	Black	32	F
7	Black	26	F
8	White	35	F
9	Black	52	M
10	White	60	M
11	Black	51	M
12	Black	51	M
13	Black	44	M
14	White	43	M

2.3.2 Method of data collection

A blood sample was drawn on each volunteer per week using a serum separating tube (SST) which contains a gel separator and a clot activator. The sample was drawn first thing in the morning; it was then centrifuged at 3000 revolutions per minute (rpm) for 10 minutes, aliquoted and stored at -80 degrees Celsius until all the samples were collected from each volunteer. When the five week duration was over all the samples from all the volunteers were thawed together and each sample per patient for each week was run in duplicate using the Elecsys 2010 Immunoanalyser, see figure 2.2. Two NT proBNP kits were used, they came from the same lot number and the

controls and calibrators that were used all came from the same lot numbers as well, we did this to reduce the analytical error of the test results.

The NT proBNP results from the Elecsys 2010 were tabulated in an excel spread sheet, indicating each sample, per individual, per week that was run in duplicate.

See table 2.1 for clarity. The NT proBNP results obtained were used to calculate the parameters required for analysis (mean, SD and CV).

Figure 2.2 Flow diagram of the sample collection method

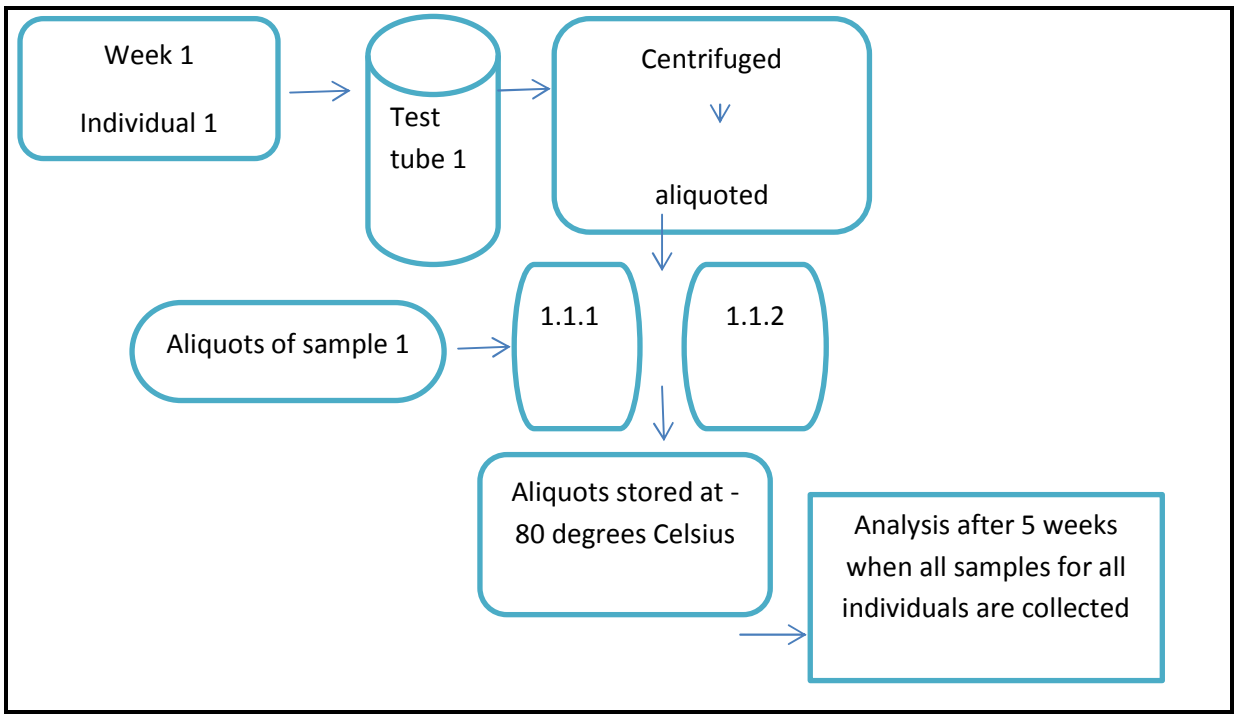


Table 2.2 Tabulation of data of the first volunteer used for analysis

Individual	Week	Sample Aliquotes	NT proBNP value (pg/ml)
1.1.1	1	1	86.55
1.1.2	1	2	66.11
1.2.1	2	1	78.95
1.2.2	2	2	66.41
1.3.1	3	1	87.32
1.3.2	3	2	62.34
1.4.1	4	1	143.30
1.4.2	4	2	112.60
1.5.1	5	1	122.00
1.5.2	5	2	65.44

2.3.3 Measuring instrument

The NT proBNP assay, is an electrochemilumeinesence immunoassay that functions on the basis of a sandwich technique, provided the assay is run on an Elecsys 2010 analyser. The assay and analyser are provided by Roche Diagnostics. The analytical range 5-35000 pg/ml, from the manufacturers' data sheet provides a reference cut-off value of 125pg/ml. Patients with values below 125pg/ml are not considered for cardiac complications, while patients with values above 125pg/ml are considered for various cardiac stresses (16). The NT proBNP assay claims stability of between 3-5 freeze thaw/ cycles (17).

The two NT proBNP reagent kits from the manufacturer Roche Diagnostics were as follows GmbH, D-68298 Mannheim. For quality control a bar coded NT proBNP calibrator (Cal Set) containing the specific information for calibration of the particular reagent lot. The controls cardiac 1 and 2 also came from the same control lot number. The NT proBNP kits were used on the Elecsys 2010 to analyse the NT proBNP analyte, it is said to have a stability of 12 months at -20 degrees Celsius (17).

2.3.4 Operation of the variables

Table 2.3 Formulation of the variables used for BV

Variation analytical & biological	Quality control technique	Formulation
Biological	Within BV	CV_i
Biological	Between BV	CV_g
Both	Imprecision	$CV_a < 0.5\ CV_i$
Both	Bias	$< 0.25[\ (CV_i)^2 + (CV_g)^2\ ]^{½}$
Both	Total error	$TE < bias + 1.65\ CV_a$ $P < 0.05$
Both	RCV	$= 2^{½} * Z * (CV_a^2 + CV_i^2)^{½}$ Z= 1.96 if P < 0.05 Z= 2.58 if P <0.01
Both	Index of individuality	$= \frac{(CV_a^2 + CV_i^2)^{½}}{CV_g}$ High > 1.4 Low < 0.6 when CVi <<CVg
Both	Total variation	$CV_T = (CV_a^2 + CV_i^2)^{½}$

2.4 Results

Table 2.4 The mean of NT proBNP over 5 weeks by sex and race (unadjusted)

Week	M/F	W/B	Observations	Mean	SD
1	Male	White	4	60.69	48.43
	Male	Black	8	21.07	7.84
	Female	White	10	97.92	108.65
	Female	Black	4	31.05	9.14
2	Male	White	4	54.19	48.96
	Male	Black	8	22.35	14.72
	Female	White	10	80.64	44.95
	Female	Black	4	33.35	5.13
3	Male	White	3	50.76	55.31
	Male	Black	8	24.85	19.43
	Female	White	10	75.83	45.87
	Female	Black	4	21.48	12.29
4	Male	White	4	68.08	54.27
	Male	Black	8	38.44	10.11
	Female	White	10	76.25	52.47
	Female	Black	4	26.75	12.39
5	Male	White	4	80.02	74.51
	Male	Black	5	37.32	18.31
	Female	White	10	74.51	43.28
	Female	Black	4	37.82	19.7
Total	Male	White	19	63.38	51.58
	Male	Black	37	28.11	15.49
	Female	White	50	8.03	62.07
	Female	Black	20	30.09	12.64

Table 2.5 Biological and Analytical variations of various groups

Groups	**Mean**	**CV**	**Between individuals**		**Within individuals**		**Analytical replicates**		*** CV_T = $(CV_A^2+CV_I^2)^{\wedge 05}$**	**RCV**
			SD_g^2	CV_g	SD_i^2	CV_i	SD_a^2	CV_a@	*CV_T	
All data	3.41	21.3	0.357	17.5	0.170	12.9	0.024	4.50	13.66	66.93
Males only	3.06	19.8	0.175	13.7	0.199	15.29	0.019	4.54	15.94	78.11
Females only	3.70	18.9	0.373	16.5	0.145	11.25	0.028	4.52	12.12	59.39
Whites only	3.68	20.8	0.570	20.5	0.080	9.15	0.033	4.94	10.40	50.96
Blacks only	3.19	19.3	0.138	11.6	0.245	16.03	0.017	4.09	16.54	81.05
All data (Correcting for race)	3.41	21.3	0.331	16.9	0.170	12.92	0.024	4.54	13.69	67.08

Table 2.6 Statistical analysis of healthy individuals

Quality control techniques	**Formula**	**Analysis**
Imprecision	Desirable analytical imprecision $CV_a < 0.5\ CV_i$	 4.5 < 6.46
Bias	Desirable Analytical Bias: Bias has to be smaller than one quarter the group biological variation $Bias < 0.25[(CV_i)^2 + (CV_g)^2]^{½}$	 $< 0.25\ (12.92^2 + 16.9^{2)½}$ Bias < 5.3
Analytical total error (TE)	TE < Bias + 1.65 CV_a (95 % Probability)	< 5.3+1.65 (4.54) TE < 12.8%
Index of individuality	$\frac{(CV_a\text{^}2+CV_i\text{^}2)^{½}}{CV_g}$	$\frac{(4.54^2+12.92^2)^{½}}{16.9}$ = 0.81
Reference Change Value (RCV)	$RCV = 2.5 * Z * (CV_A^2 + CV_I^2)^{\wedge 0.5}$	$=2.5*1.96*(4.54^2+12.92^2)^{½}$ = 67.08 = 67%

2.5 Discussion

In table 2.1 the age of the healthy individuals are shown, there is a positive relationship between NT proBNP levels and age (18-20). The relationship with age and advancing myocardial mass is a contributor to elevated NT proBNP levels (21) as well as the slowed renal clearance of NT proBNP as age and progresses, which is not easily detected by the plasma creatinine concentration (22). Age seems to have a clear effect on the metabolic clearance of the analyte (23).

$CV_a < 0.5CV_i$ defines that the desirable performance of the analyte has been achieved in all the categories of our NT proBNP data. The formula above is the original most widely accepted and very frequently used quality specification based on BV. Due to the desirable performance obtained with $CV_a < 0.5CV_i$, the amount of variability or analytical noise added to the true test result variability is between 10%-12% (2). The good precision indicated in Table 2.6 is good enough to reduce inherent variability for every individual test result. The good precision leads to greater probability of significance for changes in serial results from an individual and, therefore to better prognostic accuracy. Our acceptable noise of 10%-12% allows us to run fewer internal quality control samples per analytical run and we will be able to use less stringent quality control rules.

Bias is an important performance characteristic when using fixed limits for test interpretation, so it should be correctly interpreted. The calculated bias of < 5.3 is seen in table 2.6. Before the bias of NT proBNP is discussed further it should be explained that bias could either be positive or negative. A positive bias would give values higher than the upper limit of the reference range, which would present with many false positive readings, a negative bias would give values below the lower reference range, and this would allow for more false negative readings. The calculated bias for our NT proBNP data should be < 5.3% which can be managed by paying careful attention to quality management, change in reagent lots and re-calibrations.

In table 2.6 the calculated analytical total error allowable for NT proBNP should be < 12.7%, for which ever technology and methodology will be used to measure NT proBNP in the laboratory setting.

The data in this study indicates a RCV of 67% seen in table 2.5-2.6, for the healthy individuals studied. The RCV tells us that a patient's second NT proBNP value would have to increase by 67% from the initial value to be of any clinical significance. The RCV in this data is calculated based on CV_1 rather than on mean values. The use of CV_1 rather than mean values leads to large RCV. Thus since we use large RCV we reduce the number of false positives and any changes labelled as significant will likely be significant.

Mair*et al* suggest that only changes greater than 50% from baseline readings should be considered for prognostic decision making for heart failure patients (24). We will use our calculated RCV from the healthy individuals to analyse patients with ACS symptoms in the next chapter. Thygesen *et al*'s study also agrees with our study emphasising a shift away from population based reference intervals and that laboratories need to report RCVs established from the biological variation (25) and the analytical total error provided for the analyte; so that methods and instrumentation used to measure an analyte can provide adequate quality specifications for analysis.

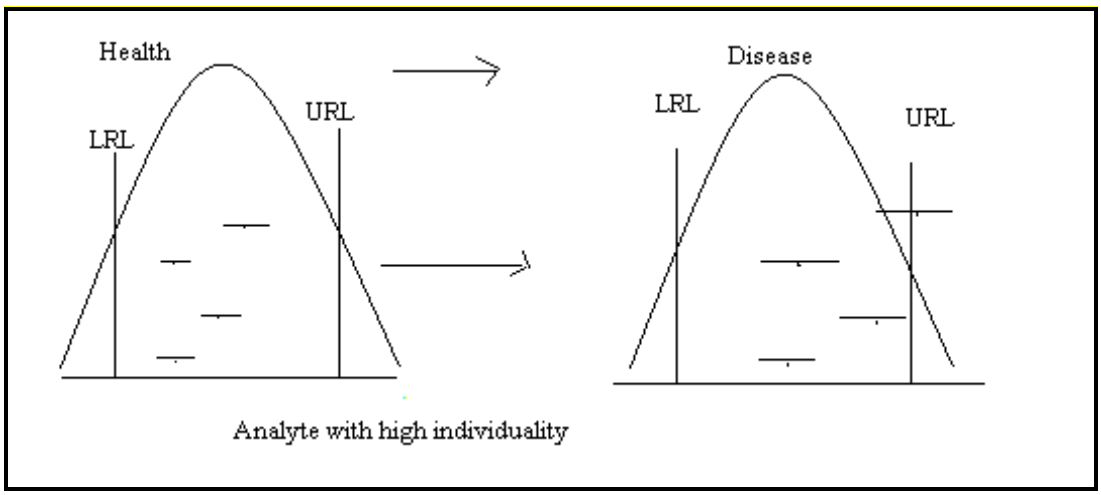
The limitation that can be discussed when looking at RCVs is that they are derived from parameters obtained from healthy individuals instead of a diseased population when they are used for the prognosis of a diseased population, but this limiting factor does not cancel out the prognostic significance that even a RCV from healthy individuals can provide.

When discussing biological individuality a few factors need to be considered. An analyte is said to have marked individuality when $CV_i < CV_g$: the analyte's individuality is decided by calculating the index of individuality. This idea came from Eugene Harris (2), who suggested that we could calculate the index of individuality as the ratio of the total within-subject variation to between-subject biological variation. The values in table 2.3 were used to calculate the index of individuality of NT proBNP, the analyte shows an index of 0.81 which implies that

population based reference intervals are not beneficial to NT proBNP. As discussed in the introduction of this chapter, for an analyte to benefit from population based reference intervals it has to present with an index of individuality greater than 1.4. Our study correlates with the work done by Araujo*et al*, on the low index of individuality of NT proBNP. They also detected an analytical total error lower than the index of individuality calculated (26).

For NT proBNP with its low index of individuality population-based reference values are of limited utility in detecting unusual results in most individuals. The influence of individuality on patient results is depicted in figure 2.3.

Figure 2.3 Analytes with a low index of individuality



Legend: LRL (lower reference limit), URL (upper reference limit)

Source: Frazer C. Biological Variation: From principles to practise. 2001 (2)

Figure 2.3 shows an analyte with high individuality measured in just four people with rather different homeostatic setting points, when people are not in good health, various factors are affected. The analyte value is affected, the range for the analyte, their mean values also deviate. Due to the individuality of these four people, even with all the above affected parameters the disease presents the homeostatic set point of most individuals will still lie within the reference interval. Only people who are lucky enough to have homeostatic set points close enough to the URL, so that when disease flags the change in parameters move the homeostatic set point outside the intervals (2). Figure 2.3 explains why BV parameters for NT proBNP need to be

considered when the analyte is used as a prognostic, marker for any cardiac related complications.

Even though this study focuses on NT proBNP there are very few other analytes that actually have an index of individuality that is greater than 1.4 and that actually benefit from population based reference intervals. This has a serious impact on result interpretation, if we continue to use population based reference intervals, diseased states will only be detected once thy are already extremely progressive.

Applying BV principles to result interpretation will not only improve the disease detection time, but it will also allow each patient to be assessed according to their own individual homeostatic setting.

The RCV that was established in this chapter on healthy individuals, without evidence of ACS, will now be used to test its true significance in the serial results of casualty patients presenting with ACS symptoms. This will indicate the reliability of our established RCV.

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Chapter 3

Use of reference change values for NT proBNP as a prognostic marker in acute coronary syndrome

3.1 Aim

- To investigate the relationship between NT proBNP and risk factors for ACS
- To evaluate the application of NT proBNP measurements in ACS risk assessment

3.2 Objectives

- To avoid pre-analytical variation
- To assess the relationship of the RCV calculated from healthy individuals with the patient outcomes listed below over a period of one year
- To measure the prognostic strength of NT proBNP for the patients presenting with ACS risk factors

3.3 Methodology

3.3.1 Study population

109 patients participated in this study as approved by the bio-statistician; we also had to take the cost of the NT proBNP kits into consideration when deciding on sample size. The study population consisted of patients who presented at the casualty units with chest pain and associated ACS symptoms, who were later admitted into the coronary units of the respective hospitals in Pretoria, Heart and Unitas hospital. *Inclusion Criteria :>* 21 years of age, both genders, all races, patients presenting with chest pain or symptoms suggestive of the ACS. *Exclusion Criteria:* Patients with abnormal renal function; poor left ventricular function (ejection fraction<45%); patients with a serum Troponin-T value of less than 0.03mg/ml.

3.3.2 Method of data collection

The standard operation procedure used to draw blood was followed by a qualified phlebotomist, a blood sample was drawn using a SST on admission at the casualty unit, and the second blood sample was drawn 24 hours following admission. The samples were centrifuged at 3000 rpm for a minimum of 10 minutes, aliquoted, and stored at -20 degrees Celsius for a period of a week. After a period of one week all the samples collected for that week were transferred to a - 80 degrees Celsius freezer where all samples were stored until analysis. It took a period of 18 months to collect the number of patients required for the study.

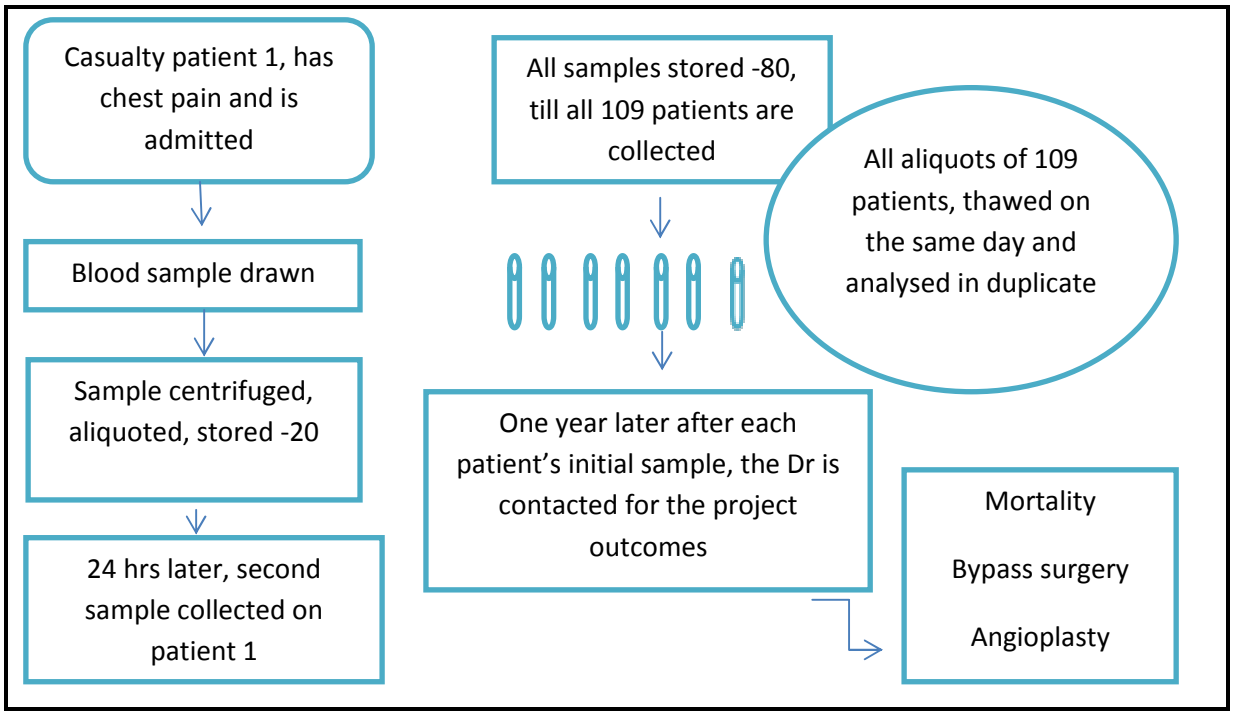
Once all the samples were collected it was time for analysis, all samples were thawed on the same day and analysed in duplicate for NT proBNP. After a period of a year for each patient, their doctor was contacted to hear if the patient had presented with either one of the outcomes: mortality, angioplasty or bypass surgery.

3.3.2.1 *Assess time of Mortality*

3.3.2.2 Probability of Angioplasty and Stenting: with the angioplasty procedure a catheter that has a balloon like tip is used to expand the narrow walls of the artery, a stent is then inserted to ensure the walls of the artery do not collapse (1).

3.3.2.3 Probability of Bypass Graft: the bypass graft requires that a portion of blood vessel from elsewhere in the body is used to graft the section of damaged area of the coronary artery; the graft bypasses the damaged area to allow easy flow of blood and therefore increases the blood flow to the heart (2).

Figure 3.1 Flow diagram of the sample collection method



3.3.3 Measuring instrument

NT proBNP serum values are determined once again by using the NT proBNP assay and the Elecsys 2010 analyser. The assay and analyser were provided by Roche Diagnostics. To ensure quality control, the NT proBNP kits, controls, and calibrator used for analysis came from the same lot numbers.

3.3.4 Statistics

Logistic regression analyses were performed as a measure of the relationship between the outcome variable and the predictor variable. The predictor variable being either a RCV that was greater and equal or smaller than the RCV that was calculated for healthy individuals and this was measured against various outcome variables. The outcome variables included; bypass surgery, angioplasty, or mortality and a relationship was assessed and then measured between the two variables. Association between and within groups was assessed using correlation and contingency table analysis.

3.4 Results

Logistic regression analysis on NT proBNP for patients with RCV >67% and their chances of Angioplasty, Bypass surgery or Death

Table 3.1 Logistic regression for NT proBNP and angioplasty

≥67% RCV	Odds Ratio	Std.Error	Z	P>Z	95% Conf.	Interval
Angioplasty	3.6	2.0	2.23	0.02	1.16	10.9

Interpretation: Patients who presented with a RCV >67% had 3.6 greater odds of requiring angioplasty compared to those with a RCV <67%. Here the RCV > 67% is significant in predicting angioplasty by 3.6.

Table 3.2 Logistic regressions for NT proBNP and bypass surgery

≥67% RCV	Odds Ratio	Std.Error	Z	P>Z	95% Conf.	Interval
Bypass Surgery	0.6	0.7	-0.45	0.65	0.057	6.01

Interpretation: Patients who presented with a RCV >67% had a 0.6 greater odds of receiving bypass surgery in comparison with patients with a RCV less than 67%, therefore no significant risk for bypass surgery is implied by the RCV.

Table 3.3 Logistic regression for NT proBNP and death

≥67% RCV	Odds Ratio	Std.Error	Z	P>Z	95% Conf.	Interval
Death	0.5	0.53	-0.64	0.52	0.06	3.92

Interpretation: Patients who present with a RCV >67% had a 0.5 greater odds of dying than those who do not present with the RCV >67%, again no significant risk is observed.

Table 3.4 Comparison of odds ratios between male & females for each of the above categories

	Exposed	Unexposed	Total	
Gender	OR	(95%Conf.Int)	M-H Weight	
0	2.1	0.28	15.9	0.6(tb)
1	2.0	0.7	5.7	2.3(tb)
Crude	2.1	0.8	5.3	
M-H combined	2.0	0.8	5.1	
Test of homogeneity (M-H)	Chi2(1)=0.006	Pr>chi2=0.9		
Test that combined OR=1;	Mantel-Haensel chi2(1)=1.95			
	Pr>chi2=0.16			

Interpretation: The Mantel-Haensezel test was used to do a statistical comparison of the odd ratios of men and woman for all the above categories and there was no significance in any of the categories when comparing gender.

Table 3.5The mean and standard deviation NT proBNP concentrations for the bypass surgery patients

Patient	1 NT proBNP (pg/ml)	2 NT proBNP (pg/ml)	67% RCV	Angioplasty	**BYPASS SURGERY**	Mortality
22	5101	6056	N	N	Y	N
23	1125	786.1	Y	Y	Y	Y
44	18908	11728	N	N	Y	N
48	174.4	205	N	N	Y	N
50	609.4	760.2	N	N	Y	N
85	6827	5072	y	y	y	N
102	825.7	1797	N	N	y	N
105	3068	3265	N	N	y	N
106	1198	875.4	N	N	Y	Y
Mean	**4204.1**	**3393.9**				
SD	**5620.1**	**3530.4**				

Interpretation: The first NT proBNP was the baseline reading, the second was the reading 24 hours later. Y means (yes) if they met the 67% RCV or if they had any of the mentioned procedures. N means (no) they did not meet the 67% RCV and they did not get a procedure.

Table 3.6 The mean and standard deviation NT proBNP concentrations for the patients that died

Patient	1 NT proBNP (pg/ml)	2 NT proBNP (pg/ml)	67% RCV	Angioplasty	Bypass surgery	**MORTALITY**
23	1125	786.1	Y	Y	Y	Y
24	8734	14148	Y	N	N	Y
25	1338	3255	Y	N	N	Y
32	44.4	388.1	Y	Y	N	Y
33	7117	7871	N	N	N	Y
34	274	747.1	Y	N	N	Y
35	1874	1351	N	Y	N	Y
36	217.4	298.3	N	N	N	Y
37	319.5	107.7	N	N	N	Y
38	32911	>35000	N	Y	N	Y
39	554.4	655.5	N	N	N	Y
40	865.7	915.6	N	Y	N	Y
41	1458	1252	N	Y	N	Y
57	4492	11239	Y	N	N	Y
81	13694	26558	N	N	N	y
82	7637	75.7	N	N	N	y
106	1198	875.4	N	N	Y	Y
Mean	**4932.6**	**4407.7**				
SD	**7948.0**	**7072.7**				

Interpretation: Here we see how many patients after our one year follow up passed away. The tabulated data also indicates that some patients had more than one of the study outcomes we looked at.

Table 3.7 The mean and standard deviation NT proBNP concentrations for the angioplasty patients

Patient	1 NT proBNP (pg/ml)	2.NT proBNP (pg/ ml)	67% RCV	**Angioplasty**	Bypass surgery	Mortality
14	9081	9448	N	Y	N	N
15	376.6	773.3	Y	Y	N	N
23	1125	786.1	Y	Y	Y	Y
28	2603	4242	Y	Y	N	N
29	242.6	940.1	N	Y	N	N
30	361.4	500.4	N	Y	N	N
31	2053	3145	N	Y	N	N
32	44.4	388.1	Y	Y	N	Y
35	1874	1351	N	Y	N	Y
38	32911	>35000	N	Y	N	Y
40	865.7	915.6	N	Y	N	Y
41	1458	1252	N	Y	N	Y
47	528	540.8	Y	Y	N	N
49	748.6	195.9	Y	Y	N	N
51	4407	9790	Y	Y	N	N
52	>35000	>35000	N	Y	N	N
53	4512	3653	Y	Y	N	N
54	123.9	613.2	Y	Y	N	N
55	23.28	1338	Y	Y	N	N
58	1109	2219	Y	Y	N	N
60	13.63	500.8	Y	Y	N	N
76	2406	1672	N	Y	N	N
85	6827	5072	y	y	y	N
87	7261	8478	N	y	N	N
90	4841	4722	N	y	N	N
101	2066	2282	N	y	N	N
Mean	**3514.5**	**2700.8**				
SD	**6482.2**	**2840.1**				

Interpretation: Angioplasty was the outcome with the largest number of patients; the standard deviation of the baseline NT proBNP reading in all our outcome groups tends to be extremely high, indicating a large deviation from the mean.

3.5 Discussion

Regression analysis was used for our study group to see if we could find any significance in the data collected on patients one year after their initial incidence. Patients presenting with NT proBNP values with RCV greater than 67% were assessed to see if they were prone to angioplasty, bypass surgery or if they died at a faster rate than those patients who did not meet the 67% RCV for NT proBNP.

In our study patients who presented with RCV >67% showed that a significant association was observed between RCV sub-group and patient outcome. In Table 3.2 we see that patients have 3.6 greater odds of requiring angioplasty if a RCV exceeding 67% is achieved. This tells us that a RCV >67% for NT proBNP can predict the probability of angioplasty by odds of 3.6 which is significant. This tells us that a RCV > 67% for NT proBNP is another added useful tool to use when clinicians decide on patients who need angioplasty.

The ACS patients present with atherosclerosis of their coronary arteries which could explain why there is a significant association with angioplasty and a RCV >67% for this study group. These patients' coronary arteries are blocked and they usually require stents to unblock their arteries. One study conducted that NT proBNP interpretations give a very similar picture about the state of the coronary disease as the interpretations after an angiogram (3).

The odds that a patient would receive bypass surgery or would die within one year if they had a RCV >67% presented with odds less than one, which implies that for mortality and predicting bypass surgery our RCV >67% is not significant and cannot be applied for our patients that were studied over a period of one year.

With regards to bypass surgery, the lack of standardised methodologies can also explain why the RCV could not predict the need for revascularization. Some doctors choose medication instead of invasive procedures; some patients' health status in combination with their age did not allow them to undergo the invasive procedures as this would have been more of a risk than putting them on medication.

Several studies conducted by Lindahl B *et al*, indicated that baseline readings of NT proBNP showed to be good predictors of mortality with ACS and coronary heart failure patients (CHF) (4-8), yet our study could not prove NT proBNP as a marker of mortality even when serial results were analysed over a period of one year.

In another study also by Jernberg T*et al* (9), conducted over a two year period, NT proBNP was not a good predictor of mortality, this shows that there is a big discrepancy between baseline NT proBNP and serial NT proBNP interpretations. Our study and the Jernberg T*et al* study that covered a period between one to two years, the serial results yields no significance with regards to mortality but the baseline NT proBNP readings seem to be good predictors of mortality. This tells us that further studies would be important to test the variances that present with serial verse baseline NT proBNP readings.

In another study also by Lindahl B *et al* (10), it was also mentioned that severe modifications to the NT proBNP levels influences drastically the important decision limits for ACS patients. This further emphases the relevance of RCVs in the analysis of NT proBNP readings. They measured NT proBNP at six months at a level of 264ng/l which had the same specificity for death as a baseline level at 722ng/l (10).

In conjunction with the previous studies, it can summarily be concluded that in order for NT proBNP to be a good predictor of mortality, one has to take into consideration the phase in which the analyte is measured. The initial acute phase, which is unstable and presents with high risk of re-infarction, shows that NT proBNP is not a relatively good predictor when compared to NT proBNP being measured in a more chronic stable phase. The chronic phase depicts a better mortality picture (10). Therefore if NT proBNP is measured in the correct phase, or if the study population is sub-categorised into acute verse chronic patients, one should be able to detect the significance of mortality which can be further investigated in future studies.

The Mantel-Haensezel test was used to carry out a statistical comparison of the odds ratios of men and woman for all the above categories and there was no significance in any of the categories (Table 3.4). Another confounding factor is that the study group

of woman investigated was not large enough to achieve a statistical conclusion that could be considered.

If we had a larger population of woman in our study we could have also been able to show that women tend to express higher levels of NT proBNP than men. Furthermore female sex hormones contribute to the genetic expression of NT proBNP and levels are also produced in the female reproductive tracts (11).

The relevance of the 67% RCV for NT proBNP informs clinicians that any value not exceeding the 67% indicates no change and that he or she should act accordingly in terms of treatment. Common practice today is for clinicians to rely on previous experiences of similar cases but this study shows that the use of RCV allows clinicians to see each patient as an individual case.

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Chapter 4

Use of NT proBNP to identify individuals with possible cardiac conditions for life insurance purpose

4.1 Aim

To investigate whether an association can be observed between abnormal ECG findings and NT proBNP levels in a population applying for life insurance.

4.2 Endpoint questions

These questions were whether:

- NT proBNP is a more reliable marker of cardiac risk than an ECG;
- Changes in NT proBNP are associated with cardiac risk factors or other impairments; and
- A raised NT proBNP in the presence of a normal ECG can be of prognostic value?

4.3 Introduction

4.3.1 Identification of risk in insurance applicants

A short medical examination known as a paramedical exam is conducted on individuals who apply for life -insurance; it is a necessary section of the application course. A qualified health professional is send by the insurance company to find out information on your health history in the form of a questionnaire and to perform a quick exam (1).

Insurance companies usually stipulate that applicants older than 50, have an ECG performed. The ECG gives information on the heart rhythms and complications that can cause tissue destruction and any other stresses on the heart. The insurance underwriter is able to get an indication of an applicant's cardiac profile from their ECG, cholesterol levels, and blood pressure readings (1).

4.3.2 Life insurance post an acute coronary event

It is common practise that if an applicant for life insurance has an acute coronary event, the underwriter ensures that the applicant's forms are not processed for a certain period. The underwriter has to wait a minimum of six months after the coronary event, be it an onset of recent angina, unstable angina, myocardial infarction, revascularisation via angioplasty, or bypass surgery to avoid the danger of having to insure such a high risk person. The six months cooling period reduces the risk for the insurance company to insure an applicant who has a high mortality chance due to myocardial infarction (2).

Previously coronary artery disease was classified as a risk with unforeseeable consequences, more recently it has become possible to assess the disease, in categories based on the intensity of the condition. A single diseased coronary vessel is not considered as severe as someone presenting with two or three diseased vessels. Then the treatment is taken into consideration whether the patient had surgery, angioplasty or just took a drug regimen, even though a publication written by Evans *et al* claims no difference in the treatment options unless the person presents with a severe case of triple vessel disease or left ventricular dysfunction. The Evans *et al* document indicated a 2% death rate yearly (2).

A condition the insurance company would not consider to insure is the risk of a person with a diseased main left coronary artery with complete stenosis that has been untreated. A second condition that is considered intensely is the left ventricular function, functionality above 50% is considered normal and insurance companies can ensure applicants with ejection fractions from 35%-50% at every costly rate, but applicants with ejection fractions less than 35% are not considered at all to be covered by insurance companies (2).

After the occurrence of a cardiac event an applicant is assessed using an exercise stress test. The parameters that are considered with a stress test are the length of the test; the maximum heart rate during the test and the capacity of work endured during the test. This information from the stress test plus information from the ECG and

sometimes even a myocardial scintigraphy is used to assess the risk of the applicant. Applicants who choose to smoke on top of their conditions are charged an extra fee, and finally a rate is concluded for an applicant (2).

4.4 Methodology

4.4.1 Population study group

Patient data was obtained from Momentum health, on applicants applying for life insurance. 106 individuals were studied, for each applicant data was provided on their (age, gender, BMI, smoking status, cholesterol levels, systolic and diastolic values, as well as their ECG readings).

4.4.2 Method of data collection

The patients would have been seen for a medical examination and ECG by a Momentum employed insurance underwriter who also collected a separate blood sample for routine testing as determined by Momentum Health. A sample of aliquoted serum on each patient was provided for NT proBNP analysis. The aliquoted samples were stored at -80 degrees Celsius, thawed on the same day and run in duplicate on the analyser. The aliquoted serum sample provided for each insurance applicant was run in duplicate on the Elecsys 2010 analyser. Tabulated on an excel spread sheet is the sample reading that was run in duplicate for each sample with all the other data provided per individual (age, gender, BMI etc.)

4.4.3 Measuring instrument

The immunoassay analyser the Elecsys 2010 was used once again to analyse the samples for NT proBNP. To reduce analytical variation the NT proBNP kits used came from the same lot number and, the same lot number was also used for control 1 and 2.

4.4.4 Statistical analysis

Following recording for the data a new NT proBNP cut-point was calculated with the ROC to assess the association between abnormal ECG findings and NT proBNP. Regression analyses were performed to determine the risk for elevated NT proBNP with abnormal ECG findings and to assess whether age and NT proBNP were independently related to abnormal ECG findings.

Table 4.1 The contribution of NT proBNP measurements to cardiac risk assessment for insurance purpose in addition to ECG evaluation.

	Penalised ECG	Normal ECG
Increased NT proBNP	X	Y
Normal NT proBNP	A	B

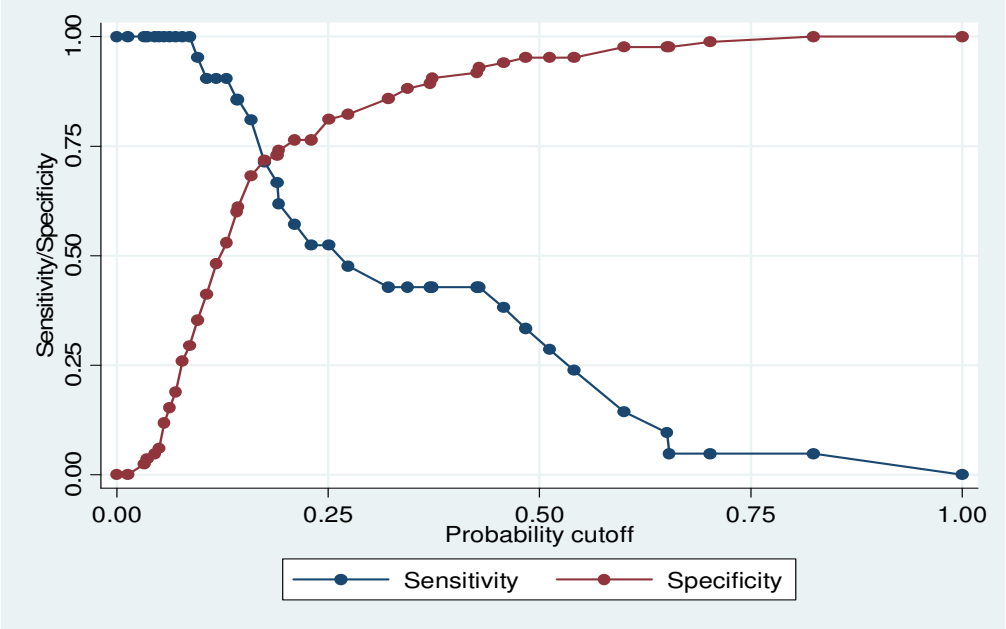
The increased and normal NT proBNP values are based on the ranges within the manufacturer's data sheet provided by Roche diagnostics. > than 125pg/ml is considered increased and <125 pg/ml is considered normal.

The odd ratio was used to compare the odds for the two groups as seen in Table 4.1- the comparison of increased NT proBNP and the penalised ECG. The odd ratio is one of a range of statistics used to assess the risk of a particular outcome (or disease) if a certain factor or exposure is present, as well as measure of association between the outcome and the exposure that presents. The odds ratio is a relative measure of risk, telling us how much more likely it is that someone who is exposed to the factor under study will develop the outcomes as compared to someone who is not exposed. Odds are a way of presenting probabilities (3). The closer the odds ratio is to one the smaller the difference in effect between the experimental intervention and the control. If the odd ratio is greater or less than one then the effects of the exposure are more or less than those of the control (4). Scalars r (rho) tetrachoric correlation coefficients for binary data were used to test for agreement between ratings.

4.5 Results

Median (range NT proBNP values for the total group were 32.43 (5-585.9) pg/mL

Figure 4.1 Determining optimal cut off values using the ROC curve



True			
Classified	D	~D	Total
+	15	24	39
-	6	61	67
Total	21	85	106
Classified + if predicted Pr (D) >=0 .16 (Optimum cut-off point with Age)			
True D defined as new NT proBNP != 0			

Sensitivity	Pr(+| D)	71.43%	
Specificity	Pr(-|~D)	71.76%	
Positive predictive value	Pr(D| +)	38.46%	
Negative predictive value	Pr(~D| -)	91.04%	
Correctly classified		71.70%	

Interpretation: With the result above, the sensitivity is 71.4% while the specificity is 71.7 and the correctly classified is about 71.7%, respectively.

If probability [Exp $^{(predicted)}$]/ [1+Exp$^{(predicted)}$] > 0.16 subject is a Case &
If probability [Exp $^{(predicted)}$]/ [1+Exp $^{(predicted)}$] < 0.16 subject is a non-case. The optimal cut off value of 0.16 presented with a more balanced sensitivity and specificity.

Table 4.2 Odd ratio analysis for elevated NT proBNP in the presence of a penalised ECG (without considering age)

Elevated NT proBNP	Odds Ratio	Std.Error	Z	P>Z	95% Conf	Interval
Penalized ECG	4.8	2.9	2.65	0.008	1.50	15.4

Interpretation: A patient having a penalised ECG has 4.8 greater odds of an elevated NT proBNP than a patient with a normal ECG.

The relationship between NT proBNP and ECG using Logistic Regression

Table 4.3 Odd Ratio Analysis for elevated NT proBNP in the presence of a penalised ECG (when considering age)

Elevated NT proBNP	Odds Ratio	Std.Error	Z	P>Z	95% Conf	Interval
Penalized ECG	2.2	1.5	1.19	0.234	0.6	8.2
Age	1.1	0.4	3.09	0.002	1.0	1.2

Interpretation: The odds ratio for an elevated NT proBNP in the presence of a penalised ECG changes drastically when age is considered, which indicates the effect age has on the relationship between elevated NT proBNP in relations to penalised ECG in general. Notice that on inclusion of age into the model, the effect of the ECG becomes non-significant. The odds suggest that there are influences of age on NT proBNP. It is important to note that for every unit change in the age, there is a 12% increase in the odds that NT proBNP will increase.

Table 4.4 Logistic regression of a penalised ECG and elevated NT proBNP in males

Elevated NT proBNP	Odds Ratio	Std.Error	Z	P>Z	95% Conf	Interval
Penalized ECG	9.8	7.6	2.98	0.003	2.2	44.4

Interpretation: In the male patients analysed there is significance or 9.8 greater odds that a man with a penalised ECG will present with an elevated NT proBNP.

Table 4.5 The logistic regression of a penalised ECG and elevated NT proBNP in males (when considering age)

Elevated NT proBNP	Odds Ratio	Std.Error	Z	P>Z	95% Conf	Interval
Penalized ECG	2.9	2.6	1.20	0.229	0.5	17.1
Age	1.1	0.1	2.71	0.007	1.0	1.3

Interpretation: Once again age has a significant influence on the odds that a male with a penalised ECG will present with an elevated NT proBNP, which reminds us of the effects of age on the NT proBNP value.

The correlation between the ECG and NT proBNP is summarised below for males and females.

Table 4.6 Correlation between the ECG and NT proBNP in males and females

Gender	Coefficient	Number	p-value	Significance
Male	0.6	79	0.005	Significant
Female	0.1	26	0.354	Not Significant

The ECG and NT proBNP are not independent of each other in males, as is evident from the correlation between the two variables ($r=0.6$; $p<0.005$). This implies that the variables can be used interchangeably.

No association was observed between NT proBNP and any of the recorded cardiovascular disease risk factors in the total group and within the gender groups.

Table 4.7Themean and standard deviation NT proBNP concentrations with a penalised ECG

	NT proBNP concentration (pg/ml)	
Patient	**ECG penalised**	**Complication**
92	20.5	ISCH
93	50.8	ISCH
94	55.37	TiNv
95	103	?
96	23.97	aNt mi
97	72	old MI
98	76.52	aNt mi
99	87	old MI
100	32.3	?
101	87.23	aNt mi
102	28.55	aNt mi
103	102.5	ISCH
104	258.1	old MI
105	169.8	ISCH
106	170	old MI
MEAN	**89.2**	
SD	**63.7**	

Interpretation: The table indicates the complications that categorised these patients into the penalised ECG category.

Table 4.8 The mean and standard deviation NT proBNP concentrations with a normal ECG

	NT proBNP concentration (pg/ml)		NT proBNP concentration (pg/ml)		NT proBNP concentration (pg/ml)
Patient number	ECG normal	Patient number	ECG normal	Patient number	ECG normal
1	74.6	31	34.79	61	28.7
2	50.05	32	19.1	62	34
3	12	33	27.4	63	64.3
4	7.5	34	59.19	64	124.8
5	8.73	35	13.8	65	14.3
6	56.1	36	103	66	78.1
7	55.7	37	18.3	67	7
8	14.34	38	58.5	68	18.8
9	27.6	39	8.99	69	17.3
10	41.4	40	5	70	64.6
11	19.6	41	69.42	71	14.69
12	14.6	42	10.87	72	17.99
13	34.5	43	13.6	73	16.6
14	15.2	44	41.6	74	55.72
15	101.4	45	91	75	56.56
16	7.8	46	72.2	76	21.9
17	32.5	47	13.2	77	37.2
18	39	48	4.4	78	7.6
19	93.5	49	20.76	79	42
20	53.3	50	23.8	80	14
21	113.4	51	24.6	81	34
22	32	52	6.6	82	31.83
23	109	53	21.51	83	80.8
24	17.24	54	70.1	84	104
25	35.8	55	20	85	73.4
26	5	56	20.11	86	7.1
27	78.6	57	8.9	87	162.98
28	17.4	58	52.72	88	171
29	95	59	12.04	89	167.7
30	36	60	25	90	258.6
				91	258.8
MEAN	**43.3**				
SD	**32.5**				

Interpretation: The majority if the clientele presented with normal ECG readings but it is clear from this table that some of these NT proBNP values are above the Roche diagnostics 125 pg/ml.

4.6 Discussion

From our data it does not seem that an elevated NT proBNP shows any pattern of correlation with any of the other cardiovascular disease risk factors that were presented by the studied population.

In Table 4.2 the data was considered without correcting for age, then age was corrected for in Table 4.3 and there is a drastic change in the odds when age is considered showing us the significant impact age has on the NT proBNP value. As stated in the results for every unit change in age there is a 12% increase in the odds that NT proBNP will increase. NT proBNP and the ECG are not independent of each other, as becomes evident after inclusion forage in the model. The lack of independence between the ECG and NT proBNP implies that there exists significant correlation between the two parameters. This implies that one may be used in place of the other, or they can be used to control or verify each other.

In Figure 4.1 there was an optimal cut off point of 0.16, which presented with a sensitivity of 71%, which is a relatively good chance of picking up any form of ischaemic stress. The specificity of 71% tells us that there are a relatively small percentage of patients that will be incorrectly identified. The 38.67% positive predictive value indicates the percentage of patients with a positive NT proBNP who are correctly diagnosed. The positive predictive value is an important measure of a diagnostic method as it reflects the probability that a positive test reflects ischemic stress.

In table 4.4 to 4.6 we considered the correlation based on gender. There was significance found in the male results when all the data was correlated. For females, it is clear that the hypothesis of independence cannot be rejected, since no significance is found. This is an indication that the use of NT proBNP in the female data may not be very reliable because of lack of evidence of association. The analysis was probably not significant due to the low number of female patients analysed. There is highly significant evidence to show that age could be a confounder in this study.

The impact of age and gender on NT proBNP levels has been reported on extensively in literature and it is clear that increasing age is positively associated with increasing NT proBNP levels (5). A recent systemic review compared the ECG to BNP and found no difference in diagnostic accuracy for left ventricular systolic dysfunction (LVSD) (5).

In conclusion determining NT proBNP concentrations may be considered to assess risk for cardiovascular disease in patients applying for insurance. The advantage of using NT proBNP concentrations is that it is a simple, fast and a valid method to detect cardiac abnormalities. Roche has recently put on the market an NT proBNP point- of-care device, it still requires validation to determine cut off points, so it can compete with the gold standard assays in use (6), but once validated will be beneficial for insurance companies.

The ECG does however provide information about previous MI, current ischaemic changes and ventricular strain. The use of the ECG however will increase the ability to discriminate between ventricular failure and cardiac ischaemia but would require an ultrasound technician, and is expensive and time-consuming (7).

4.7 References

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Chapter 5

Conclusion

5.1 Conclusion of each Hypothesis

5.1.1 Determination of the biological variation of NT pro BNP

The indices of variation that we measured for NT proBNP were RCV, index of individuality and we also looked at analytical parameters that would assist in the quality control of our method and instrumentation used. The parameters included bias, TE_a as well as the imprecision of the analyte.

These indices of variation gave us insight on NT proBNP, from the RCV calculated we could determine the percentage change that needs to be exceeded before any prognostic change can be considered. With the use of RCV we move away from population based reference intervals and it has been calculated that a RCV of 67% is required for NT proBNP, meaning that for every individual the baseline NT proBNP value will have to increase by 67% before being considered significant. The 67% NT proBNP change allows clinicians to more accurately monitor patients and use NT proBNP for prognostic purposes in patients presenting with ACS.

From the index of individuality we learnt that NT proBNP has a low index of individuality and that population based reference intervals are not beneficial for NT proBNP interpretations and RCV should be considered. The fact that NT proBNP presented with a low index of individuality is of great prognostic and diagnostic significance. The low index of individuality also indicates the misleading information provided by reference ranges whereby an analyte can still fall within the reference range of the population but is actually indicative of a diseased state for the individual. The index of individuality further indicates the importance of RCV for the majority of analytes measured in the laboratory, as very few analytes ever have an index of individuality greater than 1.4. The section explaining the index of individuality has to be clearly understood and is discussed clearly in Chapter 2.

5.1.2 Use of reference change values for NT pro BNP as a prognostic marker in acute coronary syndrome

In chapter 3 we tried to determine if the serum concentrations of NT proBNP could be used as a prognostic marker in patients presenting with ACS symptoms at a hospital casualty unit when the calculated RCV of 67 % was considered. We focused on three outcomes of which angioplasty reflected significant results when the RCV calculated was used. Mortality and bypass surgery did not reflect any significance, with regards to mortality and NT proBNP it was established that the state in which the individual is in plays a crucial role chronic verse acute as this presents with a large number of variances when it comes to result interpretation. With regards to the bypass surgery there were so many influencing factors, when it comes to invasive procedures; clinicians have to study each individual case be it the patients' age, their health status and so many other factors that made it very difficult to detect a pattern that can be statistically analysed.

5.1.3 Use of NT pro BNP to identify individuals with possible cardiac conditions for life insurance purpose

In the final chapter we found no significant difference between the ECG and NT proBNP, so either or both tests can be used. NT proBNP is a simple test that is run on patient's serum and within 20 minutes a result is available. NT proBNP measurement requires far less effort than the ECG preparation and interpretation work required. This makes NT proBNP a possible marker for insurance companies to use when detecting heart complications with their clientele.

5.2 Conclusion on the research problem

The reality is that there is an increase in complications that are cardiovascular related, mainly due to lifestyle changes that have affected the diet programs as well as the exercise regimens that a large percentage of the population follow. This large new

population of people who are presenting with symptoms that point in the direction of some type of heart ailment, need to be diagnosed correctly and secondly the best prognostic approaches needs to be taken to manage the problems at hand. The only way this can be managed correctly by the scientific community is to really have a full understanding of the markers we use to diagnose and manage certain conditions, to know how best to manipulate the results for the most accurate interpretation of a reading. That is what this study aimed to do with NT proBNP by studying the intricate details of the analyte and seeing what further information we could learn from the analyte with ACS symptomatic patients.

5.3 Study conclusions in comparison to other studies

The RCV calculated in our study corresponds well with the intra individual changes calculated in previous studies that suggested a change of between 50-66 % in serial results for NT proBNP (1). Our study also showed a positive relationship between NT

proBNP and age in chapter 2 of the dissertation which corresponds with published literature (2-4). The low index of individuality calculated for NT proBNP as well as the TE_a lower than the index of individuality corresponds with Araujo*et al*'s studies (5).

Several studies by Lindahl *et al* could show that even baseline readings of NT proBNP seemed to be good predictors of mortality, but our study could not prove this with serial result analysis (6-10).

5.4 Limitations

There was a very low number of woman in our casualty patient analysis, a larger number of woman would have allowed us to prove statistically that woman have higher levels of NT proBNP, because of the role of the female sex hormones have on the genetic expression of NT proBNP (11).

The RCV calculated came from a healthy population; if it would be possible to calculate a RCV from the diseased group of study it would yield more accurate results.

The costly NT proBNP kits did not allow us to have a larger study population which could have yielded more significant results. On the other hand it took on average 18 months to find patients at the casualty unit that met our inclusion criteria a larger study population would mean a study that prolonged for a longer duration.

5.5 Recommendations for future research

For further research on NT proBNP still focused on ACS patients, there is the option of more outcomes to extend on the three we looked at in this study. If possible a study population that reflects a more equal distribution of men to woman. Preferably the study population should be further sub-divided in patients who are either in an acute or chronic state, as this presents with many variances when it comes to result interpretations, further analysis on this would be interesting.

5.6 References

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