The Use of Hair Cortisol as a Biomarker of Chronic Stress in Patients with a recent Acute Myocardial Infarction

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by

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STATEMENT OF ORIGINAL AUTHORSHIP

The work contained in this thesis has not been submitted for any degree at any institution of higher learning. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signed by candidate

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ABSTRACT

Background

Acute Myocardial infarction (MI) or heart attack is a leading cause of death worldwide. Since an MI is a stressful life event, plasma cortisol levels are expected to increase significantly from the baseline. Cortisol and dehydroepiandrosterone (DHEA) as products of the *hypothalamic-pituitary-adrenal* (HPA)-axis have been used to diagnose endocrine disorders in serum, urine and saliva. However, these body fluids reflect short-term assessments. Hair is a promising alternative and offers several advantages over serum e.g. hair collection is painless, provides a longer detection window (days to months depending on length) and is easy to store. Recent studies confirm good correlation between hair cortisol levels and validated stress questionnaires (i.e., hair cortisol is a biomarker of stress). It is unclear whether stress is a result or cause of acute MI (i.e., chicken or egg). The primary aim of this study was to determine whether hair cortisol is higher in patients with acute MI versus healthy controls and whether the higher levels pre-date acute MI (i.e. is hair cortisol a reliable biomarker of chronic stress that predates acute MI).

Methods

This pilot study aimed to include 25 age-matched controls and acute MI cases, both 25 ST-elevation myocardial infarctions (STEMIs) and 25 non-ST-elevation myocardial infarctions (NSTEMIs). Complete versus partial coronary artery occlusion is associated with STEMIs and NSTEMIs respectively. Cortisol levels were measured in 3 cm segments of hair representing 9-12 months of growth (which would be before the heart attack in acute MI cases). The proximal 3 cm closest to the scalp, is estimated to represent 3 months before heart attack.

The samples were prepared and analyzed for cortisol concentrations using an enzyme-linked immunoassay kit. In addition, at least three strands of hair from each participant were mounted on a glass slide and subjected to Fourier-Transform Infrared (FTIR) spectroscopy.

For cortisol concentrations two-sample Wilcoxon rank-sum (Mann-Whitney) was used for within an individual comparisons and Kruskal-Wallis test was used for within group comparisons ($P<0.05$ considered significant). For FTIR data Principal Component Analysis (PCA), Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) and Partial Least Squares (PLS) models were used to compare groups.

Results

Of the intended sample size of 75, 64 participants (48 acute MI cases and 16 healthy controls) were recruited. The total hair cortisol concentration was significantly higher in acute MI cases than controls [median 23.66 (3.73-209.18) vs. 3.32 (0.37-11.24) pg/mg], ($p<0.001$), and higher in the hair of STEMI versus NSTEMI cases [35.18 (8.15-209.180 vs. 17.24 (3.73-148.22) pg/mg], ($p<0.01$). Further, cortisol levels were similar in proximal versus distal hair segments (within an individual) of STEMIs ($p>0.05$) but significantly different in those of NSTEMIs [29.64 (5.87-148.42) vs 9.94 (5.15-32.95)], ($p=0.002$).

In view of the fact that all 16 controls included were female, the analysis was repeated to include only female study participants [16 controls and 27 cases (10 STEMIs and 17 NSTEMIs)].

The total hair cortisol concentration was also significantly higher in female acute MI cases than controls [median 21.59 (3.73-209.18) vs. 3.32 (0.37-11.24)], ($p<0.001$), as well as higher in the hair of STEMI vs. NSTEMI cases [median 37.21 (8.15-209.18) vs. 14.11 (3.73-

148.22pg/mg], ($p<0.05$). Further, cortisol levels were similar in proximal versus distal hair segments of STEMIs ($p>0.05$) but significantly different in those of NSTEMIs [median 15.26 (5.91-100.41) vs. 9.94 (3.73-37.57)], ($p<0.01$). Controls had the lowest cortisol levels and there was no significant difference between proximal and distal hair within the individuals [median 3.18(1.43-7.09) vs. 2.30 (0.68-4.62) pg/mg], ($p>0.05$).

For FTIR data, OPLS-DA showed a separation between acute MI cases and controls, as well as a separation between STEMIs and NSTEMIs. Further, there was also good correlation between total cortisol concentrations and FTIR spectral data (correlation coefficient = 0.85).

Study limitations

Although not all intended participants were recruited during the study period, repeating the analysis for only female participants did not alter the findings.

Discussion and Conclusions

Hair cortisol levels were higher in acute MI cases than controls and significantly higher in STEMIs versus NSTEMIs. Further, FTIR data separated acute MI cases from controls and correlated with cortisol concentrations. Patients with Cushing Disease (CD) are reported to have a higher prevalence of thromboembolic events compared to Cushing Syndrome from adrenal sources, this is thought to be a result of high cortisol levels associated with CD. In this study, STEMIs had the highest cortisol levels which not only predated the acute MI but were unchanged in hair segments correlating with the preceding 9 months (i.e., were chronically consistently high). Interestingly, high cortisol levels also predated the acute MI but more than doubled in the proximal 3 cm hair segments (correlating with 3 months) before the heart attack in NSTEMIs.

This study suggests that based on hair cortisol concentrations as a biomarker, chronic constantly high stress predates STEMIs. Although also high in NSTEMIs it is lower than in STEMIs and more than doubles before the onset of an acute MI. The effects of chronic very high cortisol levels on the thromboembolic pathway may be to cause complete (versus partial) coronary artery occlusion in STEMIs. These results require validation in larger studies.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

APPENDICES

ABBREVIATIONS

11β-HSE – 11β Hydroxysteroid dehydrogenase

18-MEA – 18-Methyleicosanoic acid

ACE inhibitors – Angiotensin-converting enzyme inhibitors

ACTH – Adrenocorticotrophic hormone

ARBs – Angiotensin II receptor blockers

MI – Myocardial Infarction

ATR-FTIR – Attenuated total reflection-Fourier transform infrared

AVG – Arginine vasopressin

CBG – Corticosteroid-binding globulin

CD – Cushing Disease

CK-MB – Creatine kinase myocardial band

CMC – Cell membrane complex

CRF – Corticotropic hormone releasing factor

CTS – Connective tissue sheath

CU – Cuticle

CV – Coefficient of variance

CVD – Cardiovascular diseases

DHEA – Dehydroepiandrosterone

DP – Dermal papilla

ECG – Electrocardiogram

ELISAs – Enzyme-linked immunosorbent assays

FTIR – Fourier-transform infrared

HDLs – High density lipoproteins

HPA – Hypothalamic-pituitary axis

HS – Hair shaft

IR – Infrared light

LC – Liquid chromatography

LDLs – Low density lipoproteins

MC – Matrix cells

MI – Myocardial infarction

NSB – Non-Specific binding

NSTEMI – Non-ST-elevation myocardial infarction

OPLS-DA – Orthogonal Projections to Latent Structures Discriminant Analysis

PC – Precursor cells

RSC – Root sheath cells

SIMCA – Soft Independent Modelling of Class Analogy

SNS – Sympathetic nervous system

STEMI – ST-elevation myocardial infarction

TMB – Tetramethylbenzidine

VTEs – Venous Thrombo-embolic events (VTEs)

CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

Acute myocardial infarction (MI), commonly known as a heart attack, is the most common coronary artery disease and has a mortality rate of approximately 1.8 million annually (1). It accounts for about 20% of all deaths in Europe (1). Recently, there is increasing data on cardiovascular diseases (CVDs) and their risk factors in Africa (2, 3), current trends suggest that acute MI is dramatically increasing in the Sub-Saharan Africa (4, 5). The study by Moyosi et al., reported that there are approximately 20 deaths for the age category (16-64 years) and approximately 400 deaths for 65 years or older per 100 000 population in South Africa (3). Acute myocardial infarction (MI) occurs when myocardial ischemia, a diminished blood supply to the heart, exceeds a critical threshold and overwhelms the myocardial cellular repair mechanisms designed to maintain normal operating function and homeostasis, causing myocardial injury (6). The causes of acute MI could be due to interaction of several genetic, lifestyle and environmental factors. Also, MI tends to affect certain ethnic groups more than others, with diet ostensibly the major contributor (7). Techniques used to diagnose acute MI include clinical examination, electrocardiogram (ECG), biochemical and pathologic features (8). The symptoms of acute MI are variable, but the most common ones are chest pain and shortness of breath (8).

Acute myocardial infarction (MI) is reported to be a significantly stressful life event; therefore, the stress hormone cortisol and lipids are expected to increase significantly from the baseline although it is not clear whether the disease might have been caused by stress rather than vice

versa (i.e., chicken or egg) (9). Elevated levels of total cholesterol, Low Density Lipoproteins (LDLs), triglycerides and cortisol are associated with an increased risk of coronary atherosclerosis and acute MI (10, 11), the monitoring of these biomarkers could therefore possibly be used to predict and prevent heart diseases. Since cortisol levels are directly related to stress levels, the diagnosis of stress was previously evaluated using validated stress questionnaires in psychiatry (9). However, questionnaires alone are not substantial enough to postulate enough scientific evidence since they qualitative. The drawbacks of using validated stress questionnaires as a sole measure of stress are that they maybe subjective and can only date back to about 30 days (9, 12).

Cortisol or Hydrocortisone is the major glucocorticoid hormone produced in the adrenal cortex. Traditionally, cortisol is quantified from blood serum, saliva or urine (13). Saliva and serum cortisol levels vary more than urine within a 24 hour period (7, 14). The disadvantage of using these acute stress sources (serum, urine, and saliva) for assessment is that cortisol has a diurnal variation and is influenced by what the patient is experiencing at the time of collection. In the blood stream, only 3-5% of the total plasma cortisol is in its unbound or biologically active form, and able to passively diffuse into cells (15). It is believed that unbound serum cortisol enters the hair follicle through the same way that it enters the saliva, i.e. via intracellular mechanisms. Hair provides a promising alternative for evaluating cortisol and other metabolites, it also offers several advantages over serum and urine. The main advantage of using hair as a testing substrate is its ability to reflect changes in endogenous substances that occurred in previous months, depending on hair length (15). Since hair has a rich blood supply, endogenous changes, which occur in the blood would be expected to be

incorporated into the growing hair fibre. The use of hair cortisol as a long-term biomarker for stress could be of vital importance in the field of research in this regard.

Heart attacks are thought to be stressful events associated with increased cortisol levels (16). However, it is also possible that a high stress level induces an acute MI, which is difficult to confirm using cortisol levels from blood, saliva or urine (i.e., a chicken and egg situation). The diagnosis of recent (30 days) stress based on validated stress questionnaires has been shown to correlate with high cortisol levels in hair (17). Scalp hair is reported to grow at a rate of 1 cm/month (18). Therefore, it is plausible possibility to suggest that in participants with long scalp hair it may be possible to measure cortisol levels that correlate with many months previously.

The purpose of the study was to determine the feasibility of using hair cortisol as a biomarker of chronic stress that predates the onset of an acute MI. Determining hair cortisol levels that correlate with a period of more than 9 months before the acute MI could suggest the potential use of hair cortisol levels as a biomarker of chronic stress. This could mean that hair cortisol could potentially be used to identify stressed patients who could be at risk of an acute MI and justify interventions such as counselling and behaviour modification to reduce stress levels. If hair was collected before discharge in patients treated for acute MI (e.g., on day 3) then cortisol levels measured from the proximal 3 cm from the scalp (would reflect the 3 months preceding the onset of the heart attack). Depending on hair length, the distal end 9-12 cm would correlate with up to a year prior to the heart attack. Insights obtained from such a

study could help in the diagnosis of chronic stress to encourage lifestyle changes and/or treatment adjustment.

1.2. Chronic Stress

Stress refers to the body's way of responding to changes in homeostasis and it can lead to both physical and psychosocial health impairment (19). Acute stress is beneficial, as it causes the body to respond to changes through various coping mechanisms, i.e., during a fight-flight response, it releases glucose to make more fuel for cellular respiration to increase muscle activity. However, chronic stress may negatively impair health by causing prolonged stimulation of the *hypothalamic-pituitary axis* (HPA)-axis (20). The HPA axis is responsible for homeostasis of serum cortisol. However, in chronic stress serum cortisol increases and fail to return to baseline, resulting in a new (higher) set-point. Allostatic loading refers to an alteration of the homeostasis set point, for example in the case of cumulative poorly regulated acute stress, leading to a new set point of homeostasis for cortisol (Figure 1) (19). Although cortisol is the most commonly used biomarker for quantifying the allostatic load in chronic stress, there are various biomarkers which could also be evaluated e.g. triglycerides, high density lipoproteins (HDL) and catecholamines (10, 11). Furthermore, biomarkers of allostatic load could be used for quantification and prediction of cumulative health risks and may serve as indicators for detecting pre-phase of a disease (21-23).

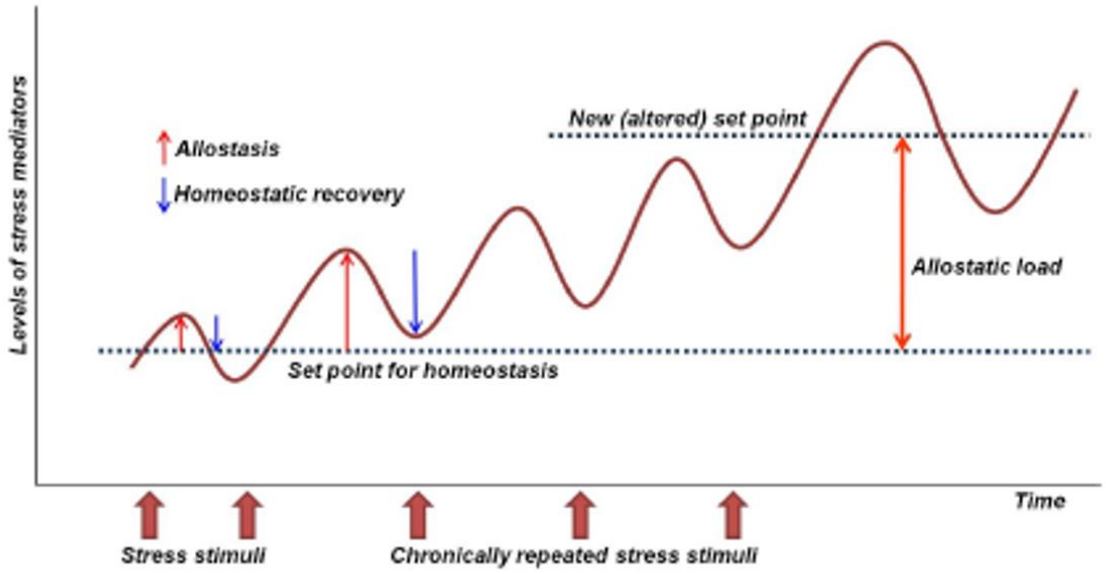


Figure 1: Stress mediators in the blood: Acute vs chronic stress**.** The stress response initially returns to baseline (set point) through a negative feedback mechanism. With repeated stress stimuli due to poorly regulated acute stress, mediators return to a new (altered - higher) set point in the blood. The difference between baseline (set point for homeostasis) and the new set point is called, allostatic load [adopted from (19)].

There is substantial evidence that chronic psychosocial stressors, including job strain, marital and financial stress, leads to increased risk of cardiovascular diseases (CVDs), including acute myocardial infarction (MI) (24). The INTERHEART study investigated associations between several psychosocial stressors with the risk of MI (24). The study included 12 461 incident cases of acute MI from 262 centers in 52 countries representing all geographic regions. They also included age-matched, sex-matched and site-matched controls free of clinical heart disease. The study showed that several elements reflecting psychosocial stress are associated

with increased risk of acute MI, which was still significant even after adjusting for other cardiovascular risk factors (such as obesity, hypertension, etc.).

1.3 Myocardial infarction (MI)

Acute MI is a condition that has recognized links to chronic stress (25-27). It is regularly accompanied by death of cardiac myocytes due to prolonged ischemia (28) as well as increased plasma levels of catecholamines, free fatty acids, and cortisol (29), resulting in serious complications such as malignant arrhythmias, left ventricular failure, and cardiogenic shock (30). The electrocardiogram (ECG) plays a vital role in the diagnostic work-up of patients with suspected MI and should be acquired and interpreted promptly after clinical presentation (31), the dynamic changes in the ECG waveforms during acute myocardial ischemia often require acquisition of multiple ECGs (8). Based on ECG, acute MI may be classified into two types**:** ST-elevation myocardial infarctions (STEMIs) and non-ST-elevation myocardial infarctions (NSTEMIs) (Figure 2A). In the normal ECG, the ST-segment lies flat and in STEMIs can be seen elevating from the baseline [Figure 2B(i)], in NSTEMIs the ST-segment lies below the baseline [Figure 2B(ii)] (32). In clinical presentation STEMIs and NSTEMIs are not easily distinguishable as patients usually present similar type of symptoms; chest pains and short breath. ST-elevation myocardial infarction (STEMI) accounts for approximately 70% of acute MI cases, these patients develop a complete occlusion of a major coronary artery which causes a full thickness damage of the supplied heart muscle (32). The NSTEMI accounts for about 30% of acute MI, patients develop a partial blockage which reduces blood flow to supplied heart muscle fibres (Figure 3) (32).

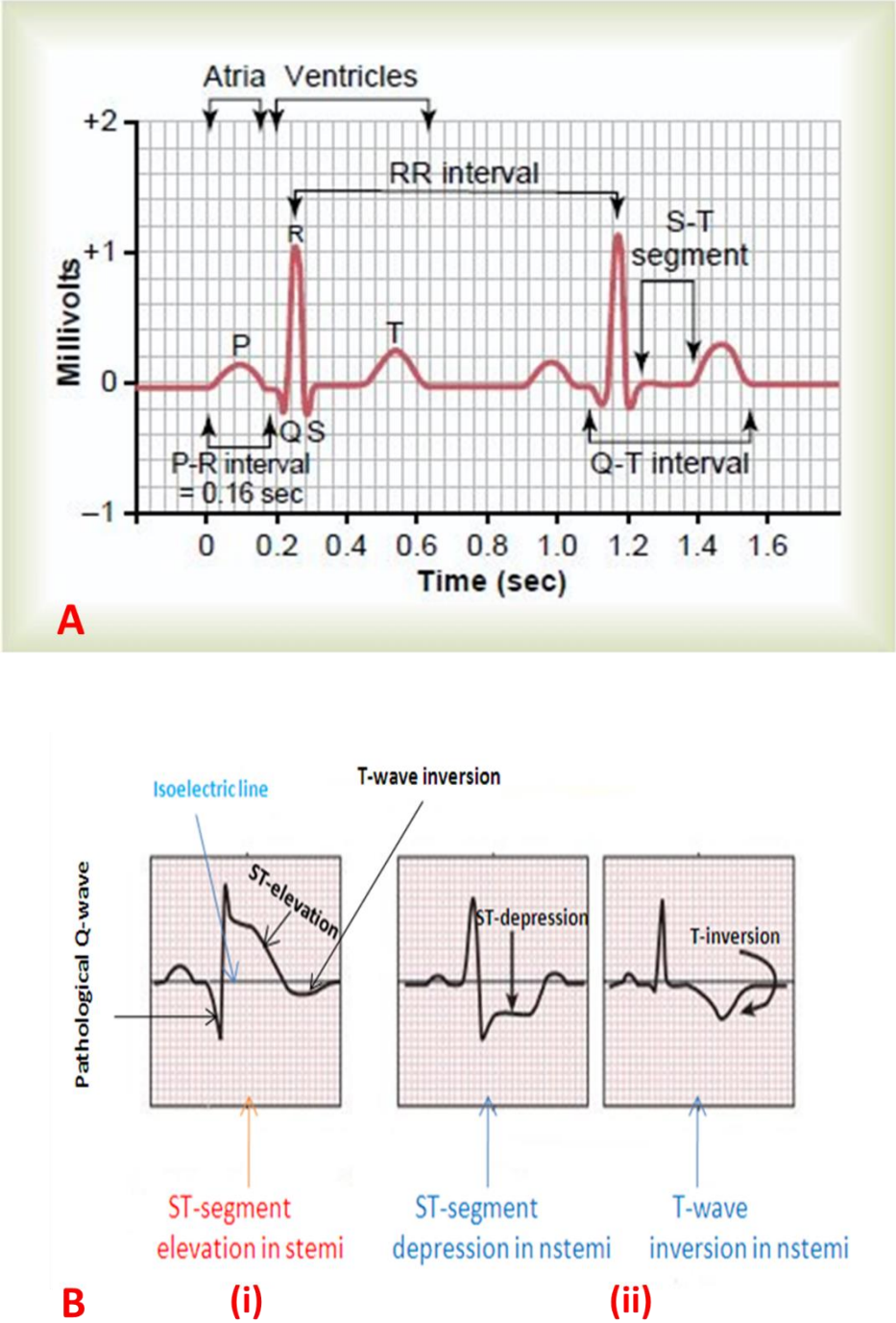


Figure 2: **(A)** Normal ECG action potentials [adopted from (33)]. **(B)** ECG showing changes in the ST segment in acute myocardial infarction [adopted from (32)].

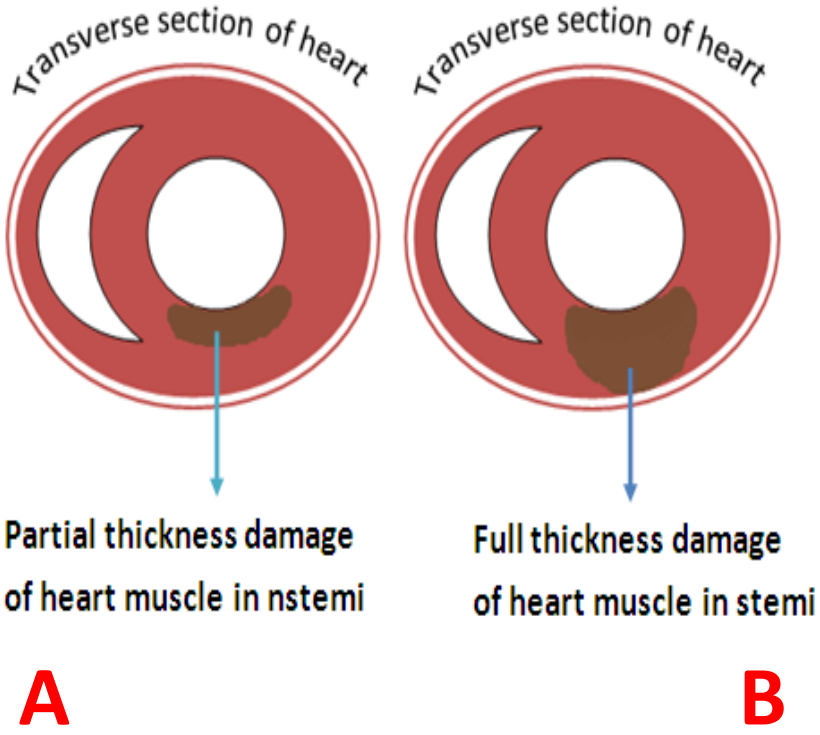


Figure 3: Transverse section of the left ventricle of the heart partial blockage to the coronary artery in **(A)** NSTEMI and **(B)** total blockage of the artery in STEMI [adopted from (32)].

1.4 Stress, Cortisol and Acute Myocardial Infarction (MI)

Stress can be defined as any stimuli that can alter homeostasis that must be counteracted by an "adaptive stress response'' (34) involving perception, interpretation, threats, etc. Although some stress is beneficial and helps the body cope (e.g., with inflammation) , if not properly managed, chronic stress may lead to both physical and physiological health impairment (35, 36), resulting in higher risk of obesity, type 2 diabetes mellitus, and cardiovascular diseases (37-39). Stress can also be the result of psychological symptoms such as anxiety, social isolation, and depression. The response to stress is mediated by the "stress hormone" cortisol, which is released in doses directly related to increasing stress levels (40). Cortisol is a lipid soluble glucocorticoid hormone which regulates a wide range of basal processes

throughout the body, including metabolism, immune response, and most importantly, it helps the body to respond to stress and to maintain homeostasis (11, 41). Cortisol does this by increasing blood sugar through gluconeogenesis, suppressing the immune system, and increasing the metabolism of fat, protein, and carbohydrates (42). The production of cortisol is housed in the cortex of the adrenal glands and then released into the blood stream where it is transported throughout the body. Cortisol is an end product of the *hypothalamic-pituitary-adrenal* (HPA)-axis (43); its secretion in response to biochemical stress may influence health and cognitive events (21, 44, 45).

1.4.1 *Hypothalamic-pituitary-adrenal* (HPA)-axis

The major regulatory systems of stress are the HPA axis and the sympathetic nervous system (SNS), (Figure 4). Activation of the HPA axis induces the release of cortisol and related steroids such as dehydroepiandrosterone (DHEA), from the adrenal cortex (46). The HPA axis is a complex set of direct influences and feedback interactions by the hypothalamus, pituitary and the adrenal glands (47). The hypothalamus initiates this hormonal pathway by secreting corticotropic hormone releasing factor (CRF) and arginine vasopressin (AVP), which in turn act upon the anterior pituitary to further propagate this signalling pathway by activating the secretion of adrenocorticotrophic hormone (ACTH) (47). The principal target for circulating ACTH is the adrenal cortex, where it stimulates glucocorticoid synthesis and secretion from the zona fasciculata (48). Under normal conditions, increased cortisol levels return to basal levels by negative feedback inhibition mechanisms via the hypothalamus, prefrontal cortex and the hippocampus. However, extreme amounts of stress may cause elevated cortisol levels or hypercortisolemia for prolonged periods which can damage the hippocampus and cortical

neurons, the main regions where feedback inhibition starts (49). Hypercortisolemia is thought to lead to structural alterations in the brain including cell apoptosis and consequent volume reductions of the prefrontal cortex and the hippocampus (50). As a result, the feedback-loop mechanism fails and cortisol levels remain high even after the stress stimuli disappear (19). This causes HPA axis dysfunction which then leads to the negative effects listed above.

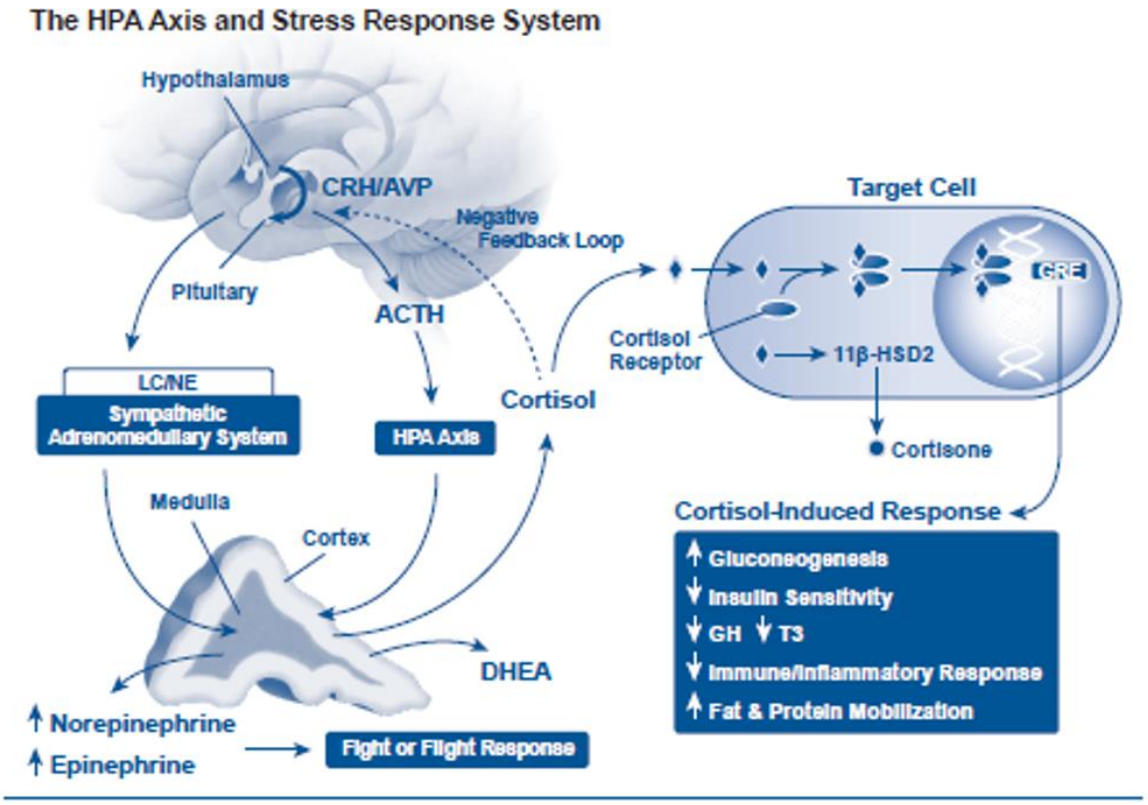


Figure 4: Schematic representation of the *hypothalamic-pituitary-adrenal* (HPA)-axis pathway (adopted from [9]). In response to stress, the HPA-axis self-regulates through negative feedback whereby elevated circulating cortisol levels lead to suppression of corticotrophin-releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH) release, thus reducing cortisol production [adopted from (20)].

Glucocorticoid hormones influence cardiovascular and metabolic regulation (51). It was recently reported in two landmark trials that blockade of the mineralocorticoid receptors is associated with the reduction of CVD complications as part of multiple biomarker strategy (52, 53). The risk of morbidity and mortality is positively correlated with elevated cortisol in acute MI patients (11). Several studies have also shown a link between chronic psychosocial stress and increased risk for developing CVD including acute MI (24-27, 54). Stress has been measured through the assessment of raised plasma corticosteroids (55). Steroid hormones that are bound non-covalently to serum proteins are biologically inactive, the unbound (free) fraction are available for uptake by target tissues (56). Zouaghi et al., investigated the total and free concentrations of steroids, mainly or partly produced by the adrenal cortex, and the corticosteroid-binding globulin (CBG) activity of sera obtained on the day of admission from patients with acute MI (57). They investigated cortisol (classical stress biomarker), ligand of CBG (an important factor in sustaining heart function), progesterone (metabolic precursor and a binding competitor for cortisol) and oestrone which appears to be involved specifically at the heart level.

Traditionally, cortisol levels were measured from blood, saliva and urine (7, 14). Zouaghi et al., study prepared sera samples by decantation of blood collected on day of admission (day 0) from a consecutive group of 34 patients with acute MI, admitted as emergency cases to the cardiac reanimation unit, over a period of 4 months (57). The results showed increased cortisol levels in patients who survived death as compared to healthy controls. In contrast the deceased MI patients did not differ in their serum cortisol levels (57) compared to healthy controls. For further validation of the variations in steroid hormones, cortisol included, the

study also analysed the amounts of unbound steroids (57) and reported that free steroids were highly increased in both survivors and the deceased. The increases were still far less significant ($P<0.001$) in deceased cases compared to survivors (57), the results suggesting that low increases of total and free blood cortisol, progesterone and oestrone predict the severity of the infarction while high levels fall in with a favour survival.

In another study, Adair et al., used serum cortisol to study the adrenal cortex response to stress of acute MI in aged cases to investigate if the degree of adrenal response was related to age or outcome (58). Mean cortisol serum concentration in old patients was compared with that in young patients, and mean serum cortisol concentration in the patients who died in the hospital was compared with that in patients who survived death (58). There was little or no difference mean serum cortisol concentrations between old and young patients but contrary to Zouaghi et al., the mean serum cortisol concentration of the deceased cases were significantly higher than those who survived (58). Higher serum cortisol and hair cortisol have been reported in patients presenting with acute MI when compared with healthy controls (11). The study reported similar cortisol levels between the first 1.5 cm and the second 1.5 cm segments, which the authors suggested would address the issue of differentiating between acute and chronic stress (11). The acute stress is reported to lasts up to 6 weeks and anything over six weeks is regarded as chronic stress.

1.5 The biology of human hair

Hair is an epithelial appendage that is found on the skin surface of almost all mammals. In animals the role of hair (or fur) includes protection and regulation of body temperatures (59). Human hair also plays an important role in sexual and social communication (59). The human skin is almost completely covered with hair except for the sole of the foot, palm of the hand, lips, and portions of external genitalia (59). The hair follicle contains the hair fiber with several surrounding structures involved in its growth, e.g., the dermal papilla which is located at and invaginates into the centre of the bulb (Figure 5). The dermal papilla has a rich blood supply and provides nourishments for the hair follicle from which new hair repeatedly cycles. Melanocytes are situated just above the dermal papilla, separated by the critical line of Auber. Melanocytes produce melanin which is responsible for hair pigmentation (60). There are two types of melanin; eumelanin predominates in black to brown hair and pheomelanin in blonde to reddish brown hair (59).

The hair growth cycle is the longest on scalp hair and is composed of three stages: anagen phase (4-6 years), catagen phase (a few weeks), and telogen phase (4-6 months). The duration of the growth cycle may influence length of hair (18), but growth can also differ depending on factors like seasons, age, gender and geographic origin. The dermal papilla is involved in important growth functions during anagen phase. The cycle starts with the longest phase, the anagen or the active growth phase. It then continues into the catagen phase, which is often termed a “transitional phase”, and finally into the telogen or the resting phase. In each cycle, a new hair shaft is formed, and the old hair is eventually shed, mostly in an actively regulated

process termed the exogen phase (61), where hair is released and falls out to accommodate the new emerging hair fibre.

The human scalp hair fibre is 50-100 µm in diameter (62), and consists of 2 or 3 layers: the outer cuticle layer, the cortex of hard-keratin and sometimes a soft-keratin medulla. The cuticle is the tough, multi-layered structure that covers the outermost surface of the hair, which is responsible for most of the resistance, stability and mechanical properties of the hair (60, 63, 64). It also contains most of the pigmentation (59). The cortex is the thickest hair layer located between the cuticle and the medulla. The medulla is not always present, in which case the cortex becomes the innermost layer.

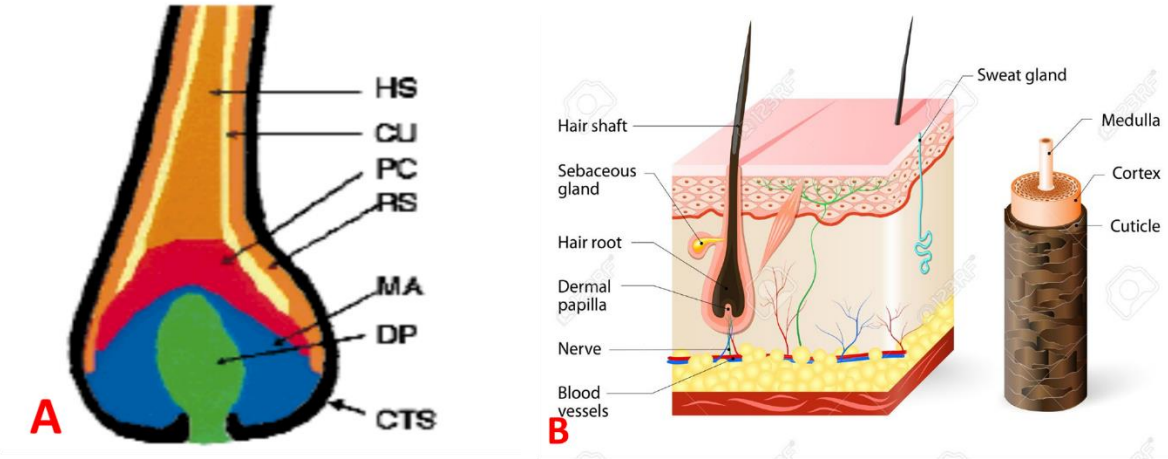


Figure 5: Schematic of the growing human hair fibre inside the follicle. The bulb-like structure located at the base of the follicle is the dermal papilla (DP, green). The DP is surrounded by undifferentiated epidermal matrix cells (MA, blue) which then give rise to precursor cells (PC, red) and, eventually, the differentiated hair shaft (HS, brown) and cuticle (CU, yellow). The hair shaft and DP are also surrounded by root sheath cells (RSC, orange) and the connective tissue sheath (CTS, black) [adopted from (65)] in **(A)** and in **(B)** Schematic of the growing human hair fibre inside dermis [adopted from (66)].

1.6 Cortisol in human hair

The use of hair as a testing substrate for monitoring chronic exposure to exogenous compounds such as drugs is increasingly gaining interest (67). Recently the correlation between validated stress questionnaires and hair cortisol levels has established hair cortisol as a biomarker of chronic stress (14, 50). Before hair cortisol analysis, cortisol was quantified from blood serum, saliva or urine. Since the levels of cortisol in the bloodstream normally fluctuates throughout day and night in a 24-hour circadian manner, which means multiple blood samples are required throughout the day to obtain an accurate measure of cortisol levels. However, collection of hair to measure hair cortisol is non-invasive and painless. Compared to serum, saliva and urine, hair is a better biological matrix for cortisol monitoring because it is easy to collect, transport and store, and is also a better repository for retrospective data [6]. Although it is not clear how cortisol is incorporated into hair, 4 mechanisms have been proposed (Figure 6): (1) through the vascular supply to the follicular cells forming the dermal papilla which generates the hair shaft (passive diffusion from blood), (2) diffusion from body secretion (sweat) during formation of the hair shaft, (3) incorporation from deep skin compartments during hair shaft formation (sebum), and (4) external environmental sources after hair shaft formation (68) e.g. through the use of cortisol containing creams, ointments, etc. (15) .

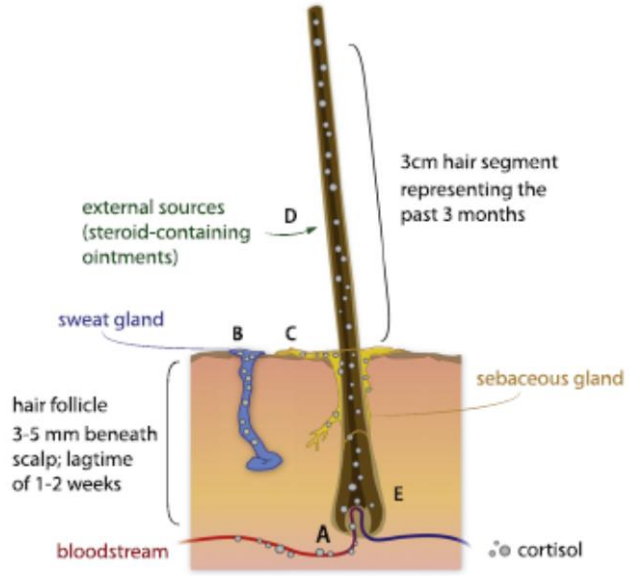


Figure 6: Schematic of proposed mechanism of cortisol incorporation into the growing human hair fibre. Cortisol incorporation from the bloodstream via passive diffusion from blood in **(A)**, diffusion from body secretions e.g. sweat gland **(B)**. From deep skin compartments during the formation of hair shaft **(C)** as well as from external sources when the hair is finally out of the epidermis **(D)** [adopted from (69)].

Cortisol incorporated through mechanisms (2) to (4) is bound on the external surface of the hair and can be removed by mild washing procedures with isopropanol. Factors which cause elevated cortisol levels need to be considered when measuring hair cortisol, such as whether a person is taking cortisol containing medications and hormone secreting tumours. Several studies have demonstrated that hair cortisol concentrations are indeed elevated in subjects undergoing significant stress compared with age-matched healthy controls or with the same subjects before stress imposition (40, 50, 70). Dettenborn et al., investigated whether hair cortisol levels differed between unemployed and employed individuals (70). Their aim was to determine whether chronic stress of being unemployed correlated with the incorporation of increased cortisol levels into hair over extended periods of time. The study showed that there

were elevated cortisol levels in hair among long-term unemployed individuals compared to healthy controls, and that hair cortisol analysis could be a useful tool for detecting differences in cumulative cortisol exposure between chronically stressed individuals and healthy controls. Another study by Dettenborn et al., hypothesized that depressed individuals would have increased hair cortisol concentrations compared with healthy controls (50). The study found that there was increased cumulative cortisol exposure of over 6 months. Including stress questionnaires in the case-control study design could improve reliability of the association between hair cortisol and stress. Furthermore, besides being the most probable repository for quantification of free, unbound cortisol, it is also unaffected by the oral intake of contraceptives (50).

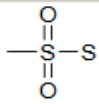
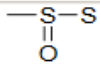
The majority of cortisol in the blood is bound to serum and only about 5-10 % is in its biologically active form. In humans this biologically active cortisol metabolizes to inactive cortisone through catalysis of 11β - hydroxysteroid dehydrogenase (11β – HSE) (71-73). How cortisol enters the hair is not well understood, but it is believed that it enters through the same mechanism by which unbound serum cortisol enters saliva via an intracellular mechanism. In saliva, the majority of cortisol remains unbound to protein, and hair cortisol is also likely to reflect the amount of free, unbound cortisol (50). The hormones are mainly delivered from the blood circulation to the capillaries of the dermal papilla, which is located inside the hair follicles [53]. Due to its stability, scalp hair is the most commonly used hair in measuring cortisol levels (less variations). For analysis, hair is sampled from the posterior vertex of the scalp and pulverised/cut into fine pieces using scissors or a bead mill. The hair cortisol is then extracted into methanol and analysed with either an enzyme-linked

immunosorbent assay (ELISA), radioimmunoassay (RIA) or liquid chromatography mass spectroscopy (LC/MS).

1.7 Fourier transform infrared (FTIR) spectroscopy and multivariate data analysis

Infrared spectroscopy is a powerful tool for analysis of biomedical samples due to the inherent chemical specificity of vibrational frequencies (74). Attenuated total reflection-Fourier-transform infrared (ATR-FTIR) spectroscopy technique which is FTIR coupled with ATR to enable the measure of variety of samples without requiring a complex sample preparation. For this study ATR-FTIR was used for characterization of molecules, using structural information collected from the surface of the hair. The technique is used for characterization of molecules, using structural information collected from the surface. Infrared light (IR) is passed through a specimen, which some is absorbed, and some is transmitted. The absorbed IR radiation corresponds to different types of bonds, and thus different vibrations of different types of bonds, and thus different functional groups, causing molecules to absorb light at different wavelengths, each having a unique fingerprint (Table 1). Background spectra should be measured before the actual analysis to make ensure that the spectra measured is from the actual specimen.

Table 1: Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy major vibrational band assignments (wavenumbers) corresponding to different types of bonds of human hair molecules [adopted from (75, 76)].

Assignments	Literature Values (cm^{-1})[152]	Previous Investigation (cm^{-1})[24]	Current Investigation (cm^{-1}) (ATR)
Amide I **80 % C=O stretch** **C-N stretch** **δ C-CN**	1690-1600	1670 1650	1669 1631-1627
Amide II **60 % C-N stretch** **40 % N-H in plane bend** **Minor contributions C-C, N-C stretch, C=O in plane bend**	1575-1480 1545 1532	1548 1534 1517	1580-1481 1520-1511
δ(C-H) deformation bend	1471-1460	1470	1461
δ(CH_2) deformation bend	1453-1443	1453	1445
δ(CH_3) deformation bend	1411-1399	1397	1392
Amide III **30 % N-H in plane bend** **30 % C-N stretch** **Contributions from C-C stretch, C=O in plane bend**	1320-1210 1260-155 1241-1231 1225	1311 1239	1322-1211 1234
 **Cystine Dioxide stretch**	1121	1121	1114
 **Cystine Monoxide stretch**	1071	1072	1071
-SO_3^-Cysteic Acid stretch	1040	1041	1037

Fourier Transform Infrared (FTIR) spectroscopy offers some advantages over standard laboratory analysis, i.e., it can provide real-time information without the use of reagents, sample are painless and cheap to collect and store. Further, the technique requires a small sample size (77, 78); for hair only, a single strand is required. The development and validation of non-invasive measurement (hair and FTIR) could be useful for diagnosis of diseases and screening, would introduce efficient clinical monitoring and permit better disease management (78). Previous studies have shown that it is possible to use FTIR to detect and measure cortisol in body fluids. Lemes *et al.*, investigated the use of FTIR spectroscopy to monitor serum cortisol levels to facilitate the diagnosis of stress in rugby players (79). This study reported the spectral region 1180 - 1102 cm^{-1} as predominantly due to cortisol (79).

The combination of FTIR spectroscopy and multivariate analysis provides improved classification and analysis of spectral data (80) as it is not always possible to visualize spectral differences in different hair samples, thus these tools are needed. The use of multivariate statistical techniques enables an efficient way of visually extracting chemical information from large spectral data, and this can be achieved through factor analysis methods like Principal Component Analysis (PCA), Partial Least Squares (PLS), Orthogonal Projections Partial to Latent Structures Discriminant Analysis (OPLS-DA) (80).

1.8 Aims & Objectives

The primary aim of this study was to investigate the potential use of hair cortisol level as a biomarker of chronic stress in patients who had sustained a recent acute myocardial infarction.

The main objectives were to:

(i) To measure Hair cortisol

a. Determine and compare the hair cortisol levels in patients with myocardial infarction (STEMIs versus NSTEMIs) and healthy controls.

b. Compare hair cortisol levels in two 3cm segments in controls, which would correlate with 3 months and 9-12 months before the acute MI in cases.

c. Review participants 3 months after the inclusion into the study to measure hair cortisol (which would reflect levels after the acute MI)

(ii) Use hair Fourier-Transformed Infrared (FTIR) Spectra

a. Compare spectra between controls and MI patients, as well as compare the spectra between STEMIs and NSTEMIs. Also compare the corresponding loading plots (also called S-line plots) cortisol region between two groups.

b. Determine whether there is a correlation between hair cortisol concentration and hair FTIR spectra.

CHAPTER 2: MATERIALS AND METHODS

2.1 Study design and Participants

This was a pilot, case-controlled study aimed at comparing hair samples from patients with acute myocardial infarction (MI) to aged matched, healthy controls. Cases were patients admitted to the hospital presenting with acute MI. The study comprised of 64 participants: 48 cases with a recent acute MI (21 STEMIs and 27 NSTEMIs) and 16 healthy controls with no current or any history of cardiovascular disease. Study participants were included and excluded based on the protocols described by Pereg *et al.,* (11) with minor modifications.

Inclusion criteria:

- Hair length of 3 cm or longer
- Age 18-72 years

Exclusion criteria:

- Glucocorticoid treatment within the last 12 months
- Diagnosis of Cushing's or Addison's disease
- Inability to sign an informed consent form

2.2 Ethical considerations

The study protocol was approved by the University of Cape Town's Faculty of Health Sciences Human Research Ethics Committee (HREC REF: 451/2016). Each participant volunteered to take part in the study and gave written informed consent.

2.3 Research site

All study participants were recruited from Groote Schuur Hospital, Cape Town, South Africa. Cases were recruited as patients admitted to the Cardiology ward at the hospital while controls were recruited as people visiting the hospital with no history of cardiovascular disease.

2.4 Hair sample collection

About 50 mg of hair was sampled from each participant; samples were taken three days after the patient's hospital admission. Attempts were made to call back all patients for another hair sample collection 3 months after the episode. Unfortunately, only seven patients responded to the call. Hair was sampled from the posterior vertex of the scalp as close to the scalp as possible (81). The hair colour, any chemical hair treatment, hair length, and participants' personal information were recorded. The hair samples were placed on aluminium foil with the root ends (proximal) and the tips (distal) clearly defined and then placed in zip-lock bags. Each sample was given a unique random number which also appeared on the consent form instead of the participant's name to anonymise the study participants. Samples were stored safely at the University of Cape Town's Hair and Skin Research Lab.

2.5 Hair sample preparation

Hair samples were sectioned into proximal and distal segments depending on the length of the hair. For acute MI cases the proximal segments were prepared in 3 cm long sections. Additionally, if the participant had hair length of greater than 12 cm, a distal segment sample of 3 cm long was also sectioned (from 9-12 cm) (Figure 7). All hair samples were washed with 3 ml isopropanol for 3 minutes. The washing procedure was repeated twice to remove external steroids and contaminants. The dry hair samples were then left to dry overnight (~ 12 hours) at room temperature. The samples were pulverized into a fine powder using Omni BeadRupture 24 (Omni International, Kennesaw, GA, USA) prior to the cortisol extraction procedure.

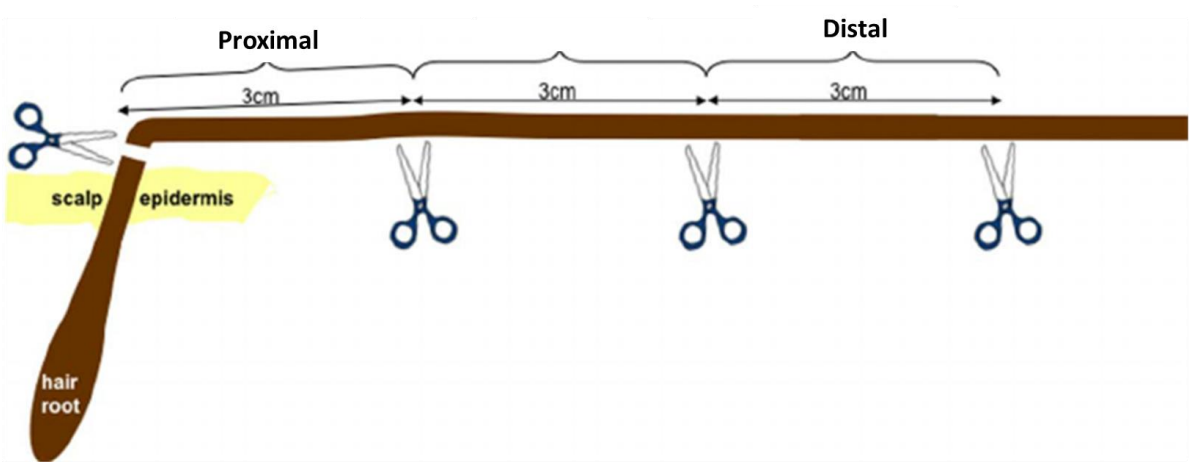


Figure 7: Schematic diagram of human hair showing the segmentation (3 cm proximal reflecting 3 months vs. 3 cm distal segment reflecting 9 months) [adopted from (82)].

2.6 Determining the amount of hair required for efficient cortisol extraction

It was important to optimize the hair cortisol extraction method using hair from the same individual. Two volunteers, one acute MI case and one healthy control, agreed to have their entire heads shaved, so that the hair could be used to optimize the cortisol extraction protocol. The cortisol extraction protocol was optimized for amount of hair used. Cortisol was extracted from 10, 25 and 50 mg hair using the procedure described in section 2.7 below. The aim was to minimize the amount of hair needed for effective and accurate levels of cortisol extraction. The first optimization was performed on a control participant as the expectation was they would exhibit lower levels of cortisol than the MI cases.

Once the amount of hair optimization yielded a minimum level of hair needed for effective extraction, a second optimization was performed. Given the expectation of high cortisol levels in the MI cases relative to the controls, an optimization was done to dilute the cortisol extract for the case participants to ensure that levels measured fell within the reference curve standard. After the cortisol was extracted, dilutions of 1:10, 1:20 and 1:50 were performed and the respective cortisol concentrations were determined.

2.7 Cortisol extraction procedure

Approximately 20 mg of pulverized hair were transferred into a 2.0 ml round bottom micro-centrifuge tube. A 1.5 ml of methanol was added to the tubes and incubated for 24 hours while shaking at 120 rpm. After incubation, samples were centrifuged for 2 minutes at 10 000 rpm to pellet the hair. The supernatant containing the cortisol extract was removed into a clean Eppendorf tube and cortisol quantified as described in section 2.8.

2.8 Measuring cortisol concentration using the enzyme-linked immunosorbent assay (ELISA)

The cortisol extract from the Eppendorf tubes were vortexed before transferring 50 µL into clean glass tubes. The extracts were dried at 38 °C under a stream of nitrogen gas. The dried sample was reconstituted with 100 µL of assay buffer and the samples were vortexed for 30s before pipetting into the plate wells. Hair cortisol concentrations were quantified using Salimetrics cortisol kit. This kit was selected because no specific hair cortisol extraction kit exists, and this kit has been used in other studies for quantification of hair cortisol. The kit was used according to manufacturers' instructions to quantify the cortisol in the hair extracts. The test principle is a competitive immunoassay kit. The technique is highly sensitive for detection and measurement of cortisol in a solution (83). Since the 96 well microtitre plate is coated with cortisol antibodies, cortisol from the test solution competes for the binding sides with cortisol conjugated to the horseradish on the plate. The samples are then incubated for some time to allow for maximum binding. After incubation, unbound components are removed with wash buffer. To avoid errors, the wells were tap dried before proceeding to the next step. The substrate tetramethylbenzidine (TMB) is then added to the microtitre plate

wells to react with the bound cortisol enzyme conjugate, the reaction produces a blue colour. The reaction is given a short incubation time before adding a stop solution which produces a yellow colour. After stopping the reaction optical densities were read at 450 nm within 10 minutes. The amount of cortisol enzyme conjugate detected reflected the amount of cortisol present in the sample.

2.9 Cortisol measurement quality control

Both a negative control (buffer only) and positive control (low control and high control standards) were used to ensure accuracy and specificity of measurement. A negative control - Non-Specific Binding (NSB) containing buffer only was used to assess any non-specific binding and this value was subtracted from zero (BO or zero standard) standards, and positive and negative controls prior to interpretation. The standard curves were plotted for further quality control validations, and the coefficients of variance (CV) across the plates and within the same plate were below 15%.

2.10 Statistical analysis of cortisol data

Results are presented as median (range), the data was analyzed for normality using STATA software (version 12.0; Stata Corporation, College Station, TX). Since the data was not normally distributed. The two-sample Wilcoxon rank-sum (Mann-Whitney) and Kruskal-Wallis tests were used because the data was not normally distributed; Wilcoxon rank-sum was used to compare the difference in hair cortisol concentrations between the segmented hair of participants and Kruskal-Wallis was used to compare cortisol concentrations between groups. P-value less than 0.05 was considered statistically significant.

2.11 Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy (ATR-FTIR)

Attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy was used to determine if there were spectral differences between hair from acute MI cases and healthy controls and if spectra could be correlated with cortisol concentration measured using ELISAs. The technique was chosen because of its simplicity and cost effectiveness over the traditional assays such as ELISAs. Either three or six strands of hair from each participant were mounted on a glass slide and subjected to ATR-FTIR analysis using a Bruker LUMOS FTIR microscope (Bruker Optics, Billerica, MA) in ATR mode. The determinant of whether three stands or six strands were used was the availability of a distal segment sample. If a distal sample was available, six stands were subjected to ATR-FTIR analysis; if a distal sample was not available then only three proximal samples were subjected to ATR-FTIR analysis. The proximal 3 cm or the distal 9-12 cm of each strand was analysed. Spectra were collected from five random points on each hair strand. Medium pressure was applied to the hair to enhance good optical contact between the sample and the crystal. Hair spectra (FTIR) were collected in the mid IR region from 450 to 4 000 cm^{-1} using 64 scans at a scan resolution of 4 cm^{-1}.

The spectra files were imported and extracted into the spectral software package OPUS version for spectral data processing. All the spectra were normalized, which divides all the absorbance values in a spectrum by the largest value in order to correct for different optical densities of the hair. The data was then imported into soft independent modelling of class analogy (SIMCA) where it was saved as spreadsheet to remove and add other components

that will enable further multivariate classification. After editing the data, the file was then saved as a SIMCA project file.

2.12 Multivariate data analysis of spectral data

The spectral data were analysed with SIMCA (MKS Umetrics) multivariate data (MVA) analysis software for identification of outliers, observation of trends and patterns in the data, and to correlate spectral data with wet chemistry data.

The importance of multivariate analysis in FTIR is well documented in the literature (79, 80, 84). Spectral data collected for FTIR analysis can be highly complex, one spectrum contains information for multiple biological materials and as a result there is an overlap for the different materials. Due to the overlap of the overlap of different molecules during experimental phase it becomes difficult to isolate relevant data. Multivariate analysis is useful in reducing this difficulties (85). Principal component analysis (PCA), Orthogonal projections to latent structures discriminant analysis (OPLS-DA) and Partial least squares (PLS) have been shown to be the types of multivariate analysis that allow for enhanced interpretation of the high dimensional spectral data of a biomaterial such as hair can produce (80).

2.12.1 Principal Component Analysis (PCA)

Principal component analysis (PCA) is a type of multivariate analysis that allows for data that has more than three dimensions to be represented visually and interpreted. It does this through converting data into a single point in space and plotting it on a graph. The method effectively handles a multitude of classes demonstrating high within-class variability (80). Principal component analysis (PCA) was chosen as an analytical technique for this study

because it is a primary tool for use in multivariate analysis and is particularly useful in the simplification of high dimensional spectral data produced from FTIR when applied to hair. This was chosen as an analytical technique for this study because it is a primary tool for use in multivariate analysis and is particularly useful in the simplification of high dimensional spectra data produced from FTIR when applied to hair (80). The transformation is done by examining the data for large variances and mapping them into a new coordinate system.

Data analysis was performed using PCA models to get an overview of the data. For interpreting the calculated variable components Y-variables, X-variables were centred. The data centring was performed to account for the shift of the origin of the Cartesian system, and the expansion or contraction of the axes. In this study, PCA looks at the hair chemical difference between high-dimensional spectral measurements from Attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy. For interpreting the calculated variable components Y-variables, X-variables were centred. The data centring was performed to account for the shift of the origin of the Cartesian system, and the expansion or contraction of the axes.

2.12.2 Orthogonal Projection to Latent Structures Discriminant Analysis (OPLS-DA)

Orthogonal projections to latent structures discriminant analysis (OPLS-DA) is a type of multivariate analysis that allows for data that has more than three dimensions (i.e., more than three variables) to be represented visually and interpreted. It is done through orthogonal transformation; it separates the predictive from non-predictive (80). It can help where PCA does not provide the class separation adequate for relevant analysis of a data set. Where PCA

relies on examining the data for the largest variances, which may or may not lie in the direction of the greatest class separation, OPLS-DA performs a transformation of PLS-DA analysis to more aggressively separate the data into classes (80). It is done through orthogonal transformation which means that variation attributed to class separation is plotted on a different axis to unrelated variation.

2.12.3 Partial Least Squares (PLS)

Partial least squares (PLS) is a type of multivariate analysis in which a linear regression model projects an observable variable and a predicted variable into a new coordinate system. This type of analysis is particularly suited to situations where the number of predicted variables far exceeds observable variables or where multicollinearity exist such as the case with FTIR spectral data from hair. In situations like these, a simple regression is not useful.

2.12.4 OPLS DA and PLS model validation

A permutation test was used for model validation on all multivariate analysis. Because PCA, OPLS-DA and PLS are analysis techniques that force underling data into groupings/ classes to aid in its understanding, it is important to validate these methods (85). A permutation test is a type of resampling that uses statistical hypothesis testing to determine whether to accept or reject the underlying analysis based on a threshold probability set at 5%. The SIMCA software performs this test by calculating a test statistic and constructs a distribution using all permutations for the test within a given model.

The permutation models is an easy way and efficient way of checking how far a model is from being a coincidence correlation by performance; it helps in assessing the validity of a

regression model (86). It is a sort of internal validation scheme, in cases where the significance of the model is doubtful it provides crucial information on whether to keep or reject the model (86).

CHAPTER 3: COMPARATIVE ANALYSIS OF HAIR CORTISOL IN SUBJECTS WITH RECENT ACUTE MYOCARDIAL INFARCTION (MI) AND HEALTHY CONTROLS

3.1 How the amount of hair required for efficient cortisol extraction was determined

It was important to determine the amount of hair needed to detect and quantify the cortisol extracted. Since the cortisol levels in hair from the control participants were expected to be low, the control participant was used for this optimization step. The cortisol was extracted from the hair and measured using the cortisol ELISA assays. The cortisol levels were determined using a cortisol reference standard. A typical reference standard curve is attached in Appendix 1.

Preliminary results for healthy controls (data not shown) displayed that it was not necessary to dilute the cortisol for the values to be within the limits of the reference standard curve. For the optimization, cortisol was extracted from 10, 25, and 50 mg of hair. The cortisol concentration was determined in the assay volume and then converted to the amount of cortisol (in picogram) per milligram of hair. The cortisol in the 50 mg sample likely reached a saturation point, as it was higher before conversion of the concentration from pg/ml (Figure 8A) to pg/mg of hair (Figure 8B). The extracts for both 10 mg and 25 mg had similar (more/less the same) concentrations in pg/mg of hair, 9.36 pg/mg and 8.63 pg/mg respectively (Figure 8B). The cortisol extracted from 50 mg of hair was lower than expected (6.02 pg/mg) (Figure 8B).

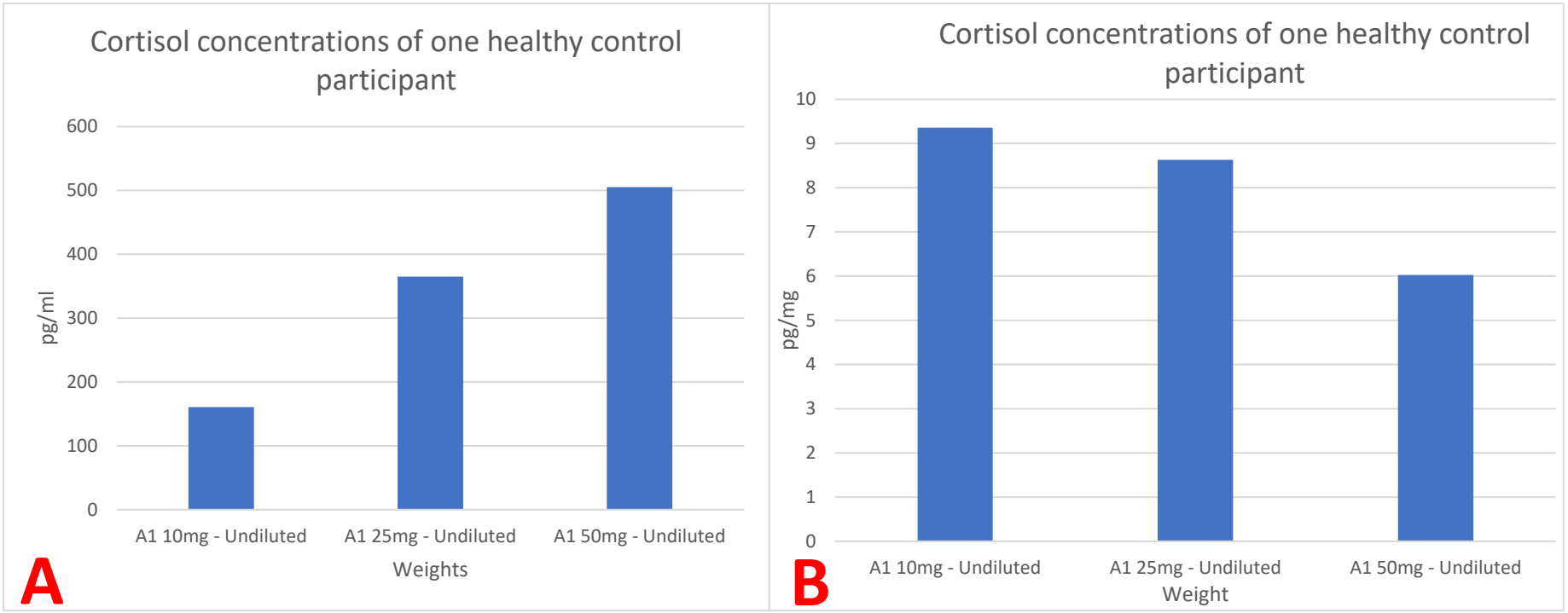


Figure 8: (A) Hair cortisol concentrations in pg/ml of one healthy control participant obtained from different weights for undiluted samples.

(B) Hair cortisol concentrations in pg/mgl of one healthy control obtained from different weights for undiluted samples.

The results indicated that it was possible to use as little as 10 mg of hair to accurately measure the cortisol concentration. It was decided to use double this amount (20 mg) in case the cortisol level was lower than the control selected for the optimization. Next it was necessary to determine the approximate dilution factor that would need to be performed for the acute MI cases where the cortisol levels were expected to be high. Cortisol was extracted from 20 mg of the acute MI case volunteer's hair and three different dilutions tested (1:10, 1:20, and 1:50). The healthy control was also repeated using 20 mg of hair, but cortisol was only measured in the undiluted sample. For the acute MI case, the 1:10 dilution was outside the limits of quantitation of the reference standard curve (too concentrated). The 1:20 and 1:50 dilutions yielded concentrations of 183.13 pg/mg and 175.95 pg/mg, respectively (Figure 9), a higher concentration than the control (6.78 pg/mg), as expected. The 20 mg worked well for both groups as the control is consistent with previous results (optimization 1 and 2), and both the 1:20 and 1:50 showed concentrations around 180.00 pg/mg. The subject's concentrations were more than 20-fold higher than the healthy control cortisol levels. Although both the 1:20 and 1:50 dilutions provided a workable level of cortisol for experimentation, the 1:20 dilution was selected as it was expected that the lower dilution would provide for less error.

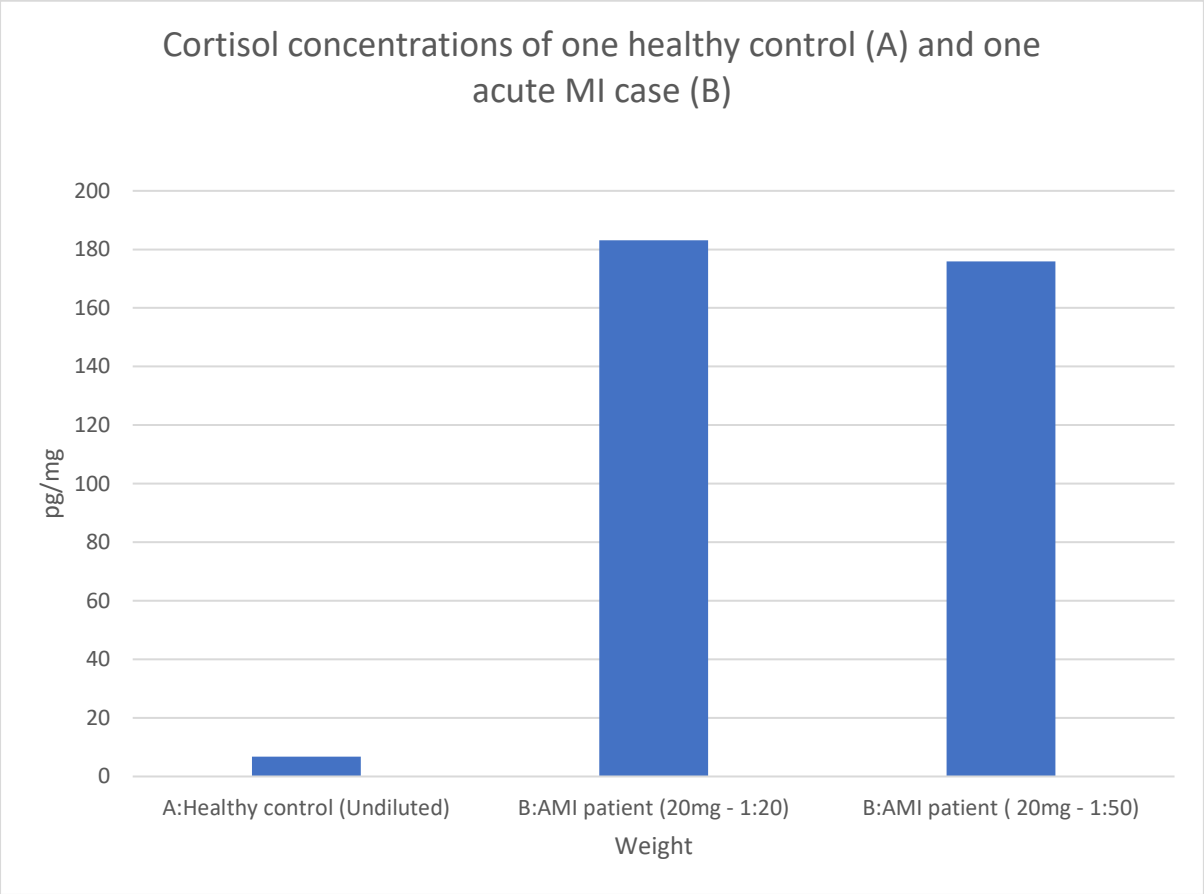


Figure 9: Hair cortisol concentrations for 20 mg samples of healthy control and acute MI participant.

3.2 Characteristics of the study population

The study was supposed to include 75 participants [50 acute MI cases (25 STEMIs and 25 NSTEMIs) and 25 healthy controls] but due to limited resources and time constraints 85% of the target was reached. The study comprised of 64 participants: 48 cases who sustained a recent acute MI, and 16 healthy controls. The acute MI cases consisted of 21 males and 27 females, and 16 female controls. Of the 48 cases; 21 (11 males and 10 females) were patients presenting with ST-elevation myocardial infarctions (STEMIs), and 27 (10 males and 17 females) were non-ST-elevation myocardial infarctions (NSTEMIs). Age distribution was tested using Shapiro-Wilk test. The mean age of the cases (acute MI patients) was 57.3±10.8, range (33-72). The mean age for controls was 43.5±8.9, range (28-58) and the overall mean age of participants was 53.9±12.0, range (28-72). The cortisol data showed a p-value of less than 0.05 when tested for normality using the Shapiro-Wilk test which suggest that the data was not normally distributed. Therefore, cortisol data is presented as median (range) (Table 2).

The average hair cortisol levels in the control group was [3.32 (0.37-11.24) pg/mg]. The average hair cortisol level in the acute MI cases was [23.66 (3.73-209.18) pg/mg]. When the acute MI group was divided into STEMIs and NSTEMIs; the NSTEMIs average cortisol level was [17.24 (3.73-148.22)], and the STEMIs average cortisol level was [35.18 (8.15-209.18) pg/mg]. The total cortisol levels between the three groups were significantly different ($p<0.001$). There was also a significant difference when STEMIs and NSTEMIs were compared to controls ($p<0.001$) as well as when STEMIs and NSTEMIs were compared to each other ($p<0.001$) (Table 2).

The overall box and whisker plot showed that there were six outliers, which might be contributing to the data being not normally distributed (Figure 10). When the box and whisker plot is divided into two groups (control group and cases), the acute MI group showed six outliers and the control group had two outliers. Due to the lower hair cortisol ranges within the control group and higher ranges within the acute MI group, the control group box and whisker plot was narrower (Figure 11A). When the acute MI group is further divided into two groups STEMIs showed four outliers (209.18, 152.09, 113.79 pg/mg and 101.45 pg/mg) and the NSTEMIs showed three outliers (148.42, 100.41 and 75.11 pg/mg) and controls had two outliers (11.24 and 9.82 pg/mg) (Figure 11B).

Table 2: Summary statistics of both the main study (males and females) and female-only analysis.

	Characteristics	STEMIs	NSTEMIs	Controls	Statistics – p-value			
					All participants	STEMIs vs NSTEMIs	STEMIs vs Controls	NSTEMIs vs Controls
All Study Participants	Mean Age (Years)	56,5±9.6	58.0±11.8	43,5±8.9	0.409	1.000 (48)	*0,001 (37)	*0,0001 (43)
	Males	11	10	0				
	Females	10	17	16				
	Cortisol Levels (Total) (pg/mg)	35.18(8.15-209.18) (29)	17.24(3.73-209.18) (42)	3,32(0.37-11.24) (27)	*0,0001	*0,0101 (71)	*0,0001 (56)	*0,0001 (69)
	Cortisol levels (Proximal) (pg/mg)	39.66(8.15-209.18) (21)	29.64(5.87-148.42) (27)	3.42(0.37-11.24) (16)	*0,0001	*0,1028 (48)	*0,0001 (37)	*0,0001 (43)
	Cortisol levels (Distal) (pg/mg)	25.18(8.29-152.09) (8)	9.94(5.15-32.95) (15)	2,30(0.68-4.62) (11)	*0,0001	0,0505 (21)	*0,0003 (19)	*0,0001 (24)
Female-only Analysis	Mean Age (Years)	54.7±11.5	60.3±11.3	43.5±8.93	0.594	0,572 (27)	*0.036 (26)	*0,000 (33)
	Females	10	17	16				
	Cortisol Levels (Total) (pg/mg)	37.21(8.15-209.18) (16)	14.11(3.73-148.22) (30)	3,32(0.37-11.24) (27)	*0,0001	*0,0358 (46)	*0,0001 (43)	*0,0001 (57)
	Cortisol levels (Proximal) (pg/mg)	40.19(8.15-209.18) (10)	22.36(8.12-148.22) (17)	3.42(0.37-11.24) (16)	*0,0001	0,1917 (27)	*0,0001 (26)	*0,0001 (33)
	Cortisol levels (Distal) (pg/mg)	29.54(8.29-152.09) (6)	9.94(3.73-152.09) (13)	2,30(0.68-4.62) (11)	*0,0001	0,0956 (19)	*0,0009 (17)	*0,0001 (24)

Note: *p < 0.05 and () Refers to the number of observations made for each group. The maximum number of observations per participant was two, however those participants who had short hair only had one observation. Most of the results are reported as median (range) except for the Mean Age (Years), the data was reported as Mean±SD as their data was normally distributed (ANOVA test was used instead of Kruskal-Wallis).

From the box and whisker plots it can be noted that the STEMIs tends to have higher cortisol levels than the NSTEMIs [35.18 (8.15-209.18) vs. 17.24 (3.73-148.22) pg/mg], ($p<0.01$), and that acute MI cases had higher cortisol levels compared to controls [23.66 (3.73-209.18) vs 3.32 (0.37-11.24) pg/mg], ($p<0.001$). Chemical treatment did not appear to affect the hair cortisol levels as there were levels over 30 pg/mg in acute MI cases that chemically treated their hair. Only three cases (acute MI patients) had chemically straightened hair. This made it difficult to make meaningful inferences on whether chemically straightening hair has an influence on hair cortisol levels.

From the STEMIs results, both genders showed variable levels as there were both found on the extremes. Most participants had cortisol levels of below 30 pg/mg, and for STEMIs only three were below 10.00 pg/mg which seemed to be the range for controls. Compared to the STEMIs, there were more NSTEMIs that were below 10.00 pg/mg. Almost, all the controls were below 10.00 pg/mg except for one which had cortisol levels of 11.24 pg/mg. The three highest extremes were all from female samples, 209.18, 152.09 and 148.2 pg/mg.

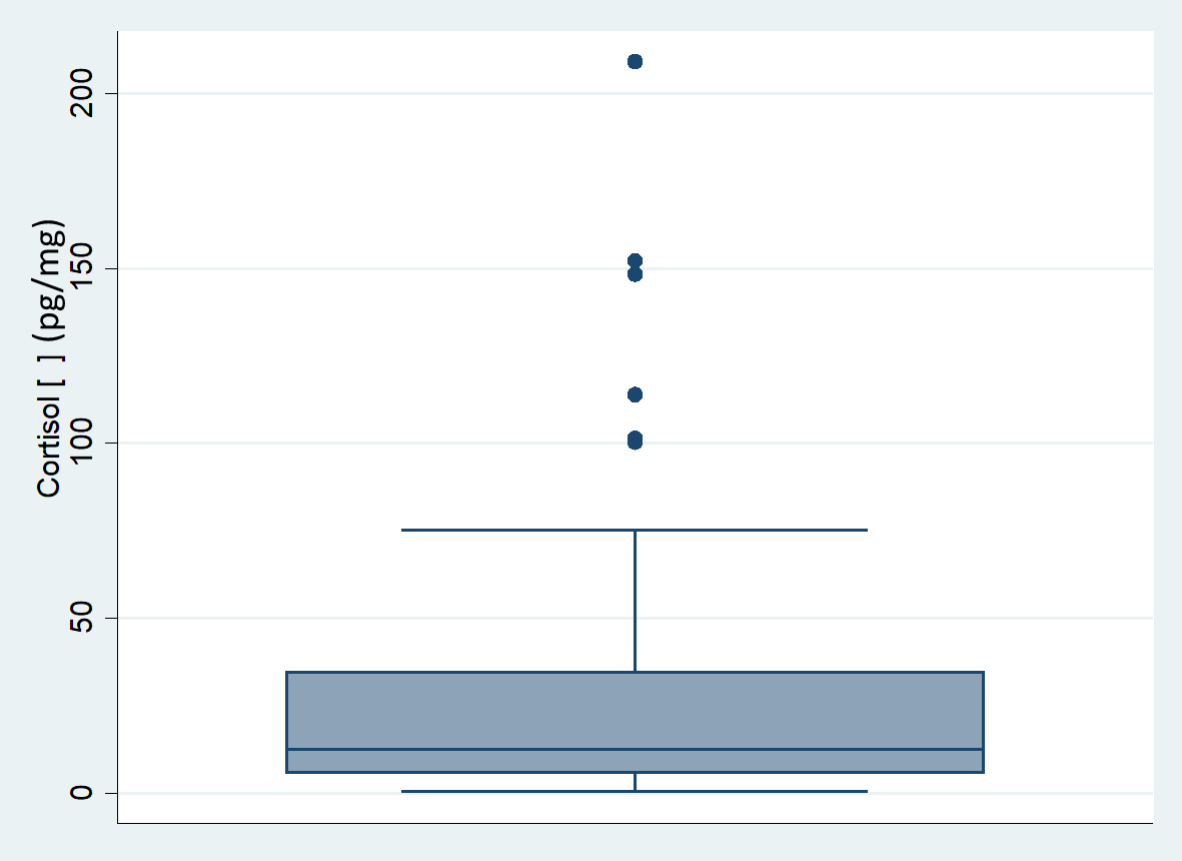
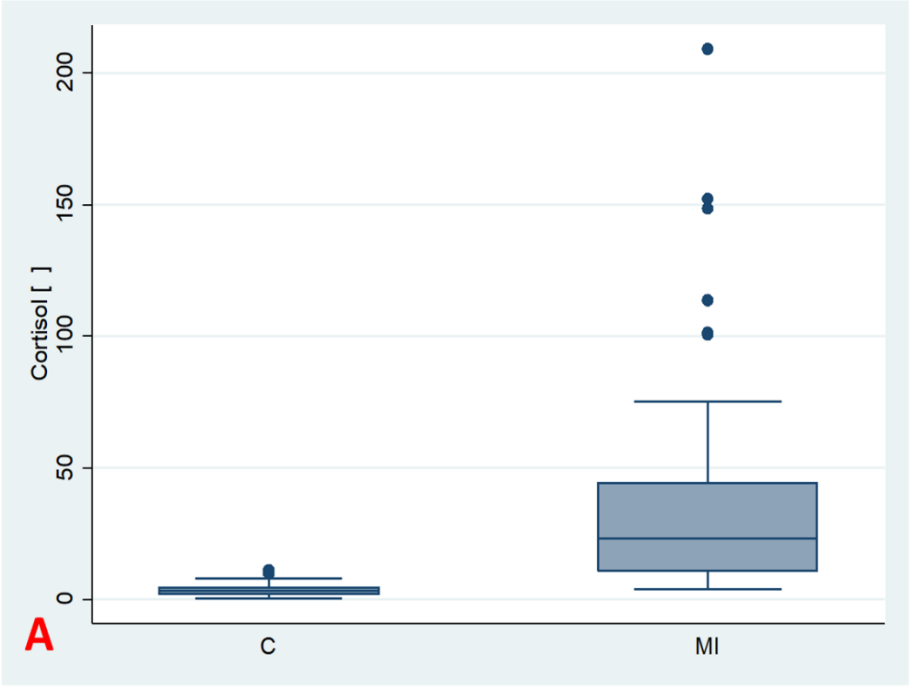


Figure 10: Box and whisker plot of hair cortisol levels of cases (acute MI patients) and healthy controls.



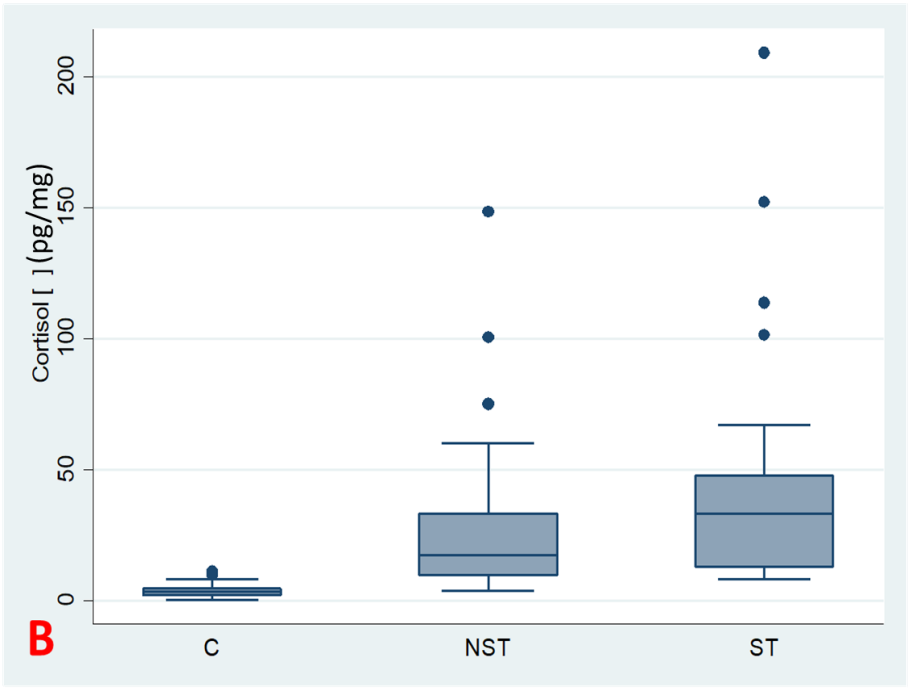


Figure 11: The overall box and whisker plot of hair cortisol levels of cases (acute MI) and healthy controls, p<0.001 in **(A)** and **(B)** Box and whisker plot of hair cortisol levels of cases (STEMIs - ST and NSTEMIs - NST) and controls.

3.3 Assessment of hair cortisol levels 3 months prior to the event

The cases were recruited soon after they were admitted to the hospital (on day 3). The proximal 3 cm hair sample is expected to reflect the hair cortisol levels up to 3 months before the heart attack. Kruskal-Wallis test was used to compare the cortisol levels between the three groups (STEMIs, NSTEMIs and controls) 3 months before the heart attack. The p-value was 0.0001, suggesting significant statistical difference between the three groups. Both STEMIs and NSTEMIs were significantly different as compared to healthy controls [39.66 (8.15-209.18) and 29.64 (5.87-148.42) vs. 3.42 (0.37-11.24) pg/mg], ($p<0.001$). There was no significant difference in proximal segment hair cortisol levels between STEMIs and NSTEMIs [39.66 (8.15-209.18) vs. 29.64 (5.87-148.42) pg/mg], ($p>0.05$).

3.4 Assessment of long-term cortisol levels (9-12 months versus 3 months prior to heart attack and 3 months after heart attack versus 3 months prior heart attack)

In most cases hair cortisol levels from the proximal segment, which represents the hair cortisol levels 3 months before the heart attack were higher than the distal segment hair cortisol levels, and since controls showed lower levels [3.32 (0.37-11.24) pg/mg] compared to the cases [23.66 (3.73-209.18) pg/mg], controls were excluded to determine whether there was an increase in hair cortisol levels between the two segments (proximal vs. distal). There was a significant difference in hair cortisol levels between proximal and distal segments prior to the heart attack [31.69 (5.87-148.42) vs. 16.01(5.15-152.09) pg/mg], ($p<0.01$). And when the controls hair cortisol levels between proximal and distal were compared there was no significant difference [3.32 (0.37-11.24) vs. 2.30 (0.68-4.62) pg/mg], ($p>0.05$). The above results represent the total cortisol levels in proximal segments (i.e., even the ones that did not have the distal segments were included).

The comparison of cortisol levels in proximal versus distal hair (within the same individuals) segments representing before the heart attack revealed no significant difference in healthy controls [3.18 (1.43-7.09) vs. 2.30 (0.68-4.62) pg/mg], ($p>0.05$) (Figure 12A); there was no significant difference in STEMIs between the proximal and distal segments respectively [37.74 (8.15-113.79) vs. 25.18 (8.29-152.09) pg/mg], ($p>0.05$) (Figure 12B), but there was significant difference in NSTEMIs between the proximal and distal segments respectively [22.36 (5.91-100.41) vs 9.94 (3.73-37.58) pg/mg], ($p<0.01$) (Figure 12C). Error bars represents the standard deviation of each participant's data set.

Only seven patients returned for hair sample collection 3 months after the heart attack, five were STEMIs and two were NSTEMIs. In the STEMIs (5 cases) the cortisol levels in the proximal 3 cm after the acute MI dropped to less than half of that of before heart attack [35.18 (13.00-113.79) vs. 14.27 (9.93-34.32) pg/mg], ($p<0.05$). Of the two NSTEMIs that returned after heart attack, one showed increased cortisol levels and one had declined hair cortisol levels.

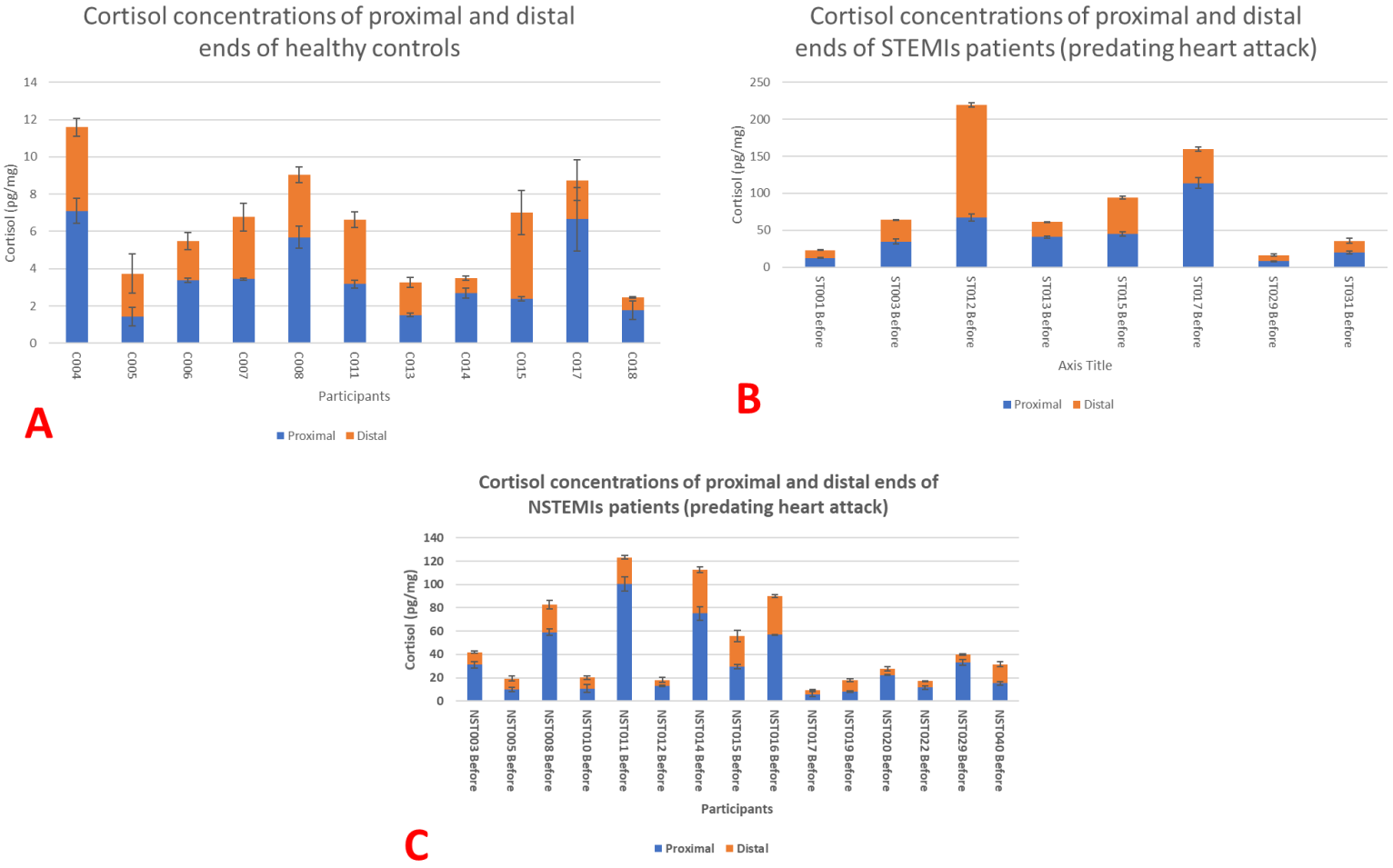


Figure 12*:* In all participants: The cortisol concentrations between proximal and distal hair were the same in healthy controls **(A)** (p = 0.155) (11) and STEMI patients (8) **(B)** (p=0.484) but significantly different in NSTEMIs patients (15) **(C)** (p= 0.002).

3.5 Characteristics of the study population (Female-only analysis)

The study was supposed to include 75 participants (50 MI patients: 25 STEMIs and 25 NSTEMIs and 25 healthy controls) but due limited resources and time constraints this was not met. Participants recruitment had to be terminated after enrolling 43 participants: 27 cases who sustained a recent acute MI and 16 healthy controls. All participants were females for this section of the study: of the 27 cases, 10 were patients presenting with STEMIs and 17 with NSTEMIs. Age distribution was tested using Shapiro Wilk test. The mean age of the cases (acute MI patients) was 54.7±11.58, range (33-70). The mean age for controls was 43.5±8.9, range (28-58) and the overall mean age of participants was 52.7±12.7, range (28-72).

The cortisol data showed a p-value of less than 0.0001 when tested for normality, which suggest that the data was not normally distributed (Shapiro-Wilk test). Therefore, cortisol results were presented as median (range). The average hair cortisol levels in the control group was [3.32 (0.37-11.24) pg/mg]. The average hair cortisol level in the acute MI cases was [21.59 (3.73-209.18) pg/mg]. When the acute MI group was divided into STEMIs and NSTEMIs; the NSTEMIs average cortisol level was [14.11 (3.73-148.22)], and the STEMIs average cortisol level was [37.21 (8.15-209.18) pg/mg]. The total cortisol levels between the three groups were significantly different ($p<0.001$). There was also a significant difference when STEMIs and NSTEMIs were compared to controls ($p<0.001$) as well as when STEMIs and NSTEMIs were compared to each other ($p<0.05$) (Table 2).

3.6 Assessment of hair cortisol levels 3 months prior to the event (Female-only analysis)

Kruskal-Wallis test was used to compare the cortisol levels between the three groups (STEMIs, NSTEMIs and controls) 3 months before the heart attack. The p-value was 0.0001, suggesting significant statistical difference between the three groups. Both STEMIs and NSTEMIs were significantly different compared to healthy controls [40.19 (8.14-209.18) and 22.36 (8.12-148.42) pg/mg] vs. [3.42 (0.37-11.24) pg/mg], ($p<0.001$). There was no significant difference between STEMIs compared to NSTEMIs [40.19 (8.14-209.18) vs. 22.36 (8.12-148.42) pg/mg], ($p>0.05$).

3.8 Assessment of long-term cortisol levels (9-12 months versus 3 months prior to heart attack – Female-only analysis)

In most cases hair cortisol levels from the proximal segment, which represents the hair cortisol levels 3 months before the heart attack were higher than the distal segments hair cortisol levels, and since controls showed lower levels [3.32 (0.37-11.24) pg/mg] compared to the cases (21.59 (3.73-209.18) pg/mg], controls were excluded to determine whether there was an increase in hair cortisol levels between the two positions (proximal vs. distal). There was enough statistical difference in immunoassays cortisol levels between cases before proximal and distal segments [29.77 (5.91-209.18 vs. 10.95 (3.73-152.09) pg/mg], ($p<0.05$), STEMIs vs. NSTEMIs, respectively. The above results represent the total cortisol levels for proximal segments (i.e., even the ones that did not have the distal segments were included).

Wilcoxon signed ranked test was used for comparisons of the cortisol levels between the proximal and distal segments. There was enough statistical difference in immunoassays cortisol levels between the three groups (STEMIs, NSTEMIs and controls) proximal and distal segments ($p<0.01$). The comparison of cortisol levels in proximal versus distal hair segments before heart attack revealed no significant difference in healthy controls [3.18 (1.43-7.09) vs. 2.30 (0.68-4.62)], ($p>0.05$), (Figure 13A); there was also no significant difference in STEMIs [40.72 (8.15-113.79 vs. 25.18 (8.29-152.09) pg/mg], ($p>0.05$), (Figure 13B). Lastly, there was significant difference in NSTEMIs [15.26 (5.91-100.41 vs. 9.94 (3.73-37.57) pg/mg], ($p<0.05$), (Figure 13C). Error bars represents the standard deviation of each participant's data set.

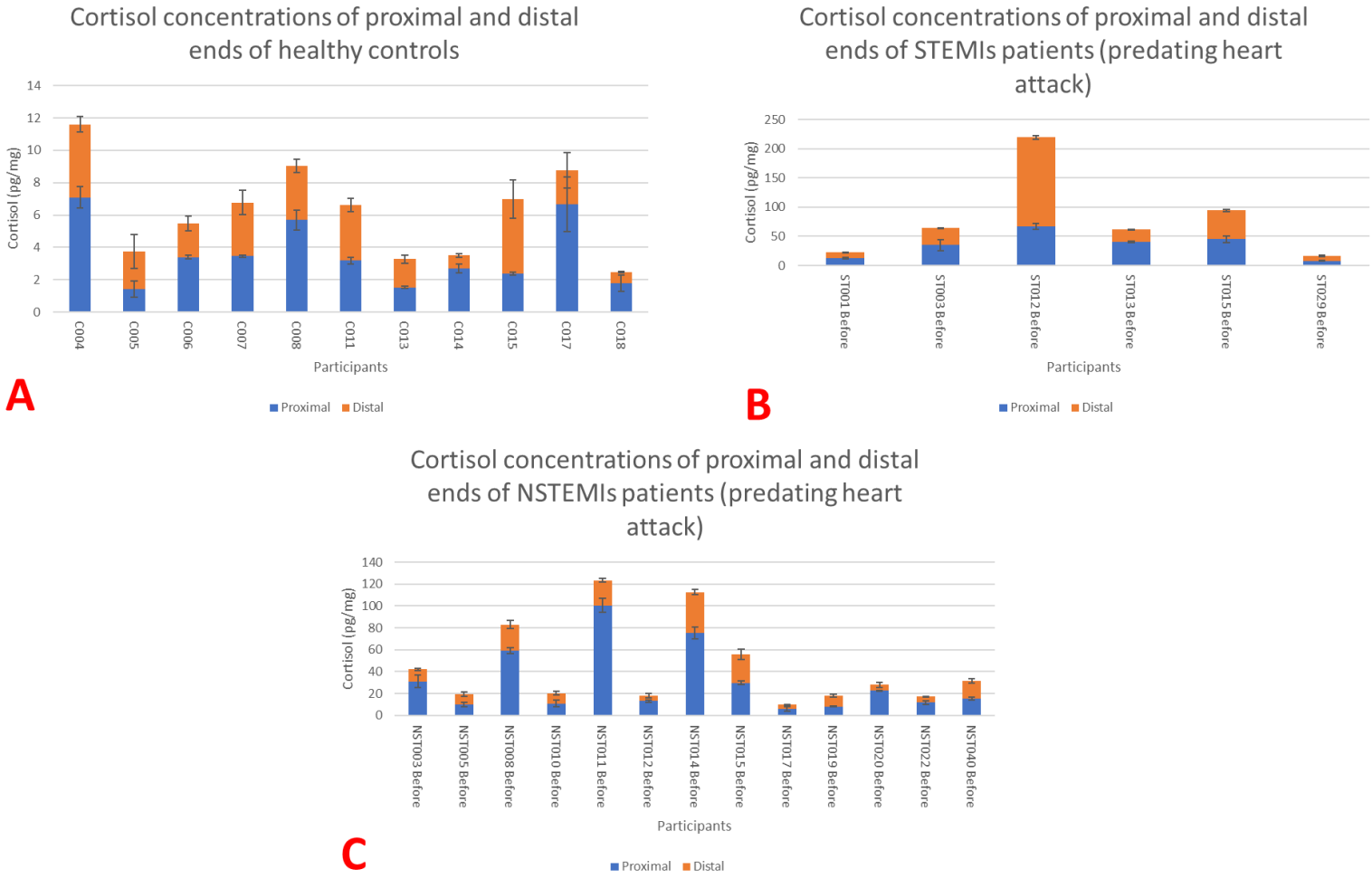


Figure 13: In females: The cortisol concentrations between proximal and distal hair were the same in healthy controls (11) **(A)**. (p=0.155) and STEMI patients (6) **(B)**. (p=0.917) but significantly different in NSTEMIs patients (13) **(C)** (p=0.006).

CHAPTER 4: INVESTIGATING THE USE OF ATTENUATED TOTAL REFLECTION–FOURIER-TRANSFORM INFRARED (ATR-FTIR) SPECTROSCOPY TO DIFFERENTIATE BETWEEN HAIR OF SUBJECTS WITH MI AND HEALTHY CONTROLS

4.1 Introduction

Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy is a technique used for chemical characterization of molecules, using structural information collected from the surface. Infrared light (IR) is passed through a specimen, some of IR radiation is absorbed and some of it is transmitted, and the absorbed IR radiation corresponds to stretching and bending of different types of bonds, and thus corresponds to different functional groups (Figure 14). Certain molecules absorb light at different wavenumber in mid-infrared region (450-4000 cm^{-1}). Background spectra should be measured before the actual analysis to ensure that the spectra measured is from the actual specimen. Fourier-transform infrared (FTIR) spectroscopy offers certain advantages over standard laboratory analysis (wet chemistry data); it can provide real-time information without the use of reagents and the technique requires smaller sample sizes (77, 78), only a single strand is required for hair analysis.

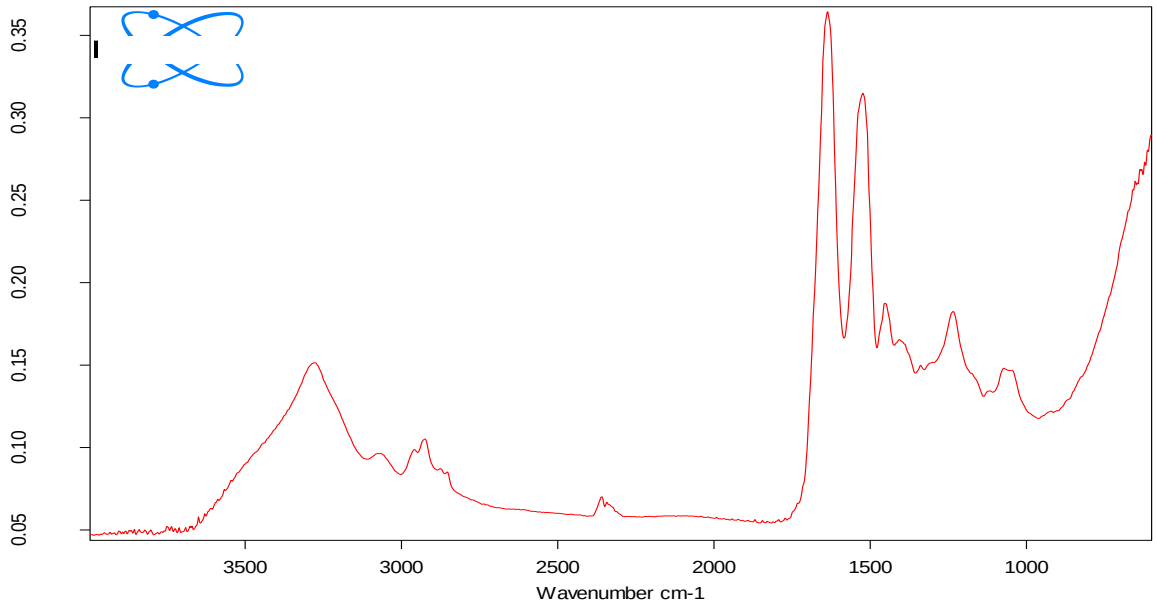


Figure 14: A single hair spectra obtained from ATR-FTIR showing peaks representing different molecular components (higher concentrations are represented by high intensities).

The aim of this chapter was to determine whether it was possible to differentiate between ATR-FTIR hair spectra of cases with acute MI and healthy controls. Spectral data were collected from the hair strands and analysed with multivariate (MVA) data analysis software for identification of outliers, observation of trends and patterns in the data, and correlating spectral data with wet chemistry data. The analysis involved a workflow to investigate the spectral differences of participants and determine the correlation of the spectra and measured hair cortisol levels of participants. Firstly, raw spectra were assessed with different principal component analysis (PCA), followed by discrimination of groups by Orthogonal Projections to Latent Squares Discriminant Analysis (OPLS-DA) and lastly the spectra were correlated with the measured cortisol levels from immunoassay analysis. The 3 cm proximal segments were used for sections that included healthy controls (Chapter 4.2, 4.3 and 4.5). Fifteen spectra were collected from each participant [i.e., 5 spectra per participant

corresponding to 5 data points from each hair strand (total of 3 strands per participant)]. The models were then internally validated through permutation testing (to check whether the models were valid or not).

4.2 Principal component analysis (PCA)

The spectral data was subjected to PCA to get a general overview and observe any patterns on the spectral data of acute MI cases and healthy controls (spectral range; 736.059 - 3627.28cm^{-1}). Since all controls were females with dyed hair, PCA model was fitted based on females with dyed hair to avoid variations in spectral data. The PCA model was generated to get a general overview of the trends relating to acute MI cases and control groups (Figure 15A). The control group spectra clustered together while acute MI cases clustered into two groups. One group clustered next to the controls (with some overlap) and the other group was completely separated from the two. Three PCA components were calculated which explained 84.1% of the variation in the spectral data. The greatest fraction of spectra variation (R2X), was explained by the first component, R2X[1] = 65.2%, which is a clear indication that the spectra had a lot of unwanted variations (i.e., noise).

The same PCA model was then coloured according to the three groups; STEMIs, NSTEMIs and healthy controls. The control group was clustering next to most of the NSTEMIs, and the STEMIs were clearly separated from the controls except for the few that overlapped with the clustered NSTEMIs and controls (Figure 15B). The spectral profiles for the STEMIs overlapping with the controls were compared with the STEMIs that completely separated from the

controls (Figure 15C). This was done by randomly selecting a single observation from each cluster (completely separated vs. overlapping STEMI spectra), the results showed in a blue spectral profile (from the overlapping side) and in black (from the separated side). The spectra from the overlapping side shows lower intensities in the region 800-1600 cm^{-1}. This is an interesting observation as the intensity of the spectrum is related to the concentration of molecules indicating lower concentration of relevant molecules in this region (peak area/concentration). The same approach was followed for the NSTEMIs (Figure 15D). Lower spectral intensities were also observed between data taken from the group clustering with controls (Black spectra) and the group clustered with the STEMIs (Blue spectra).

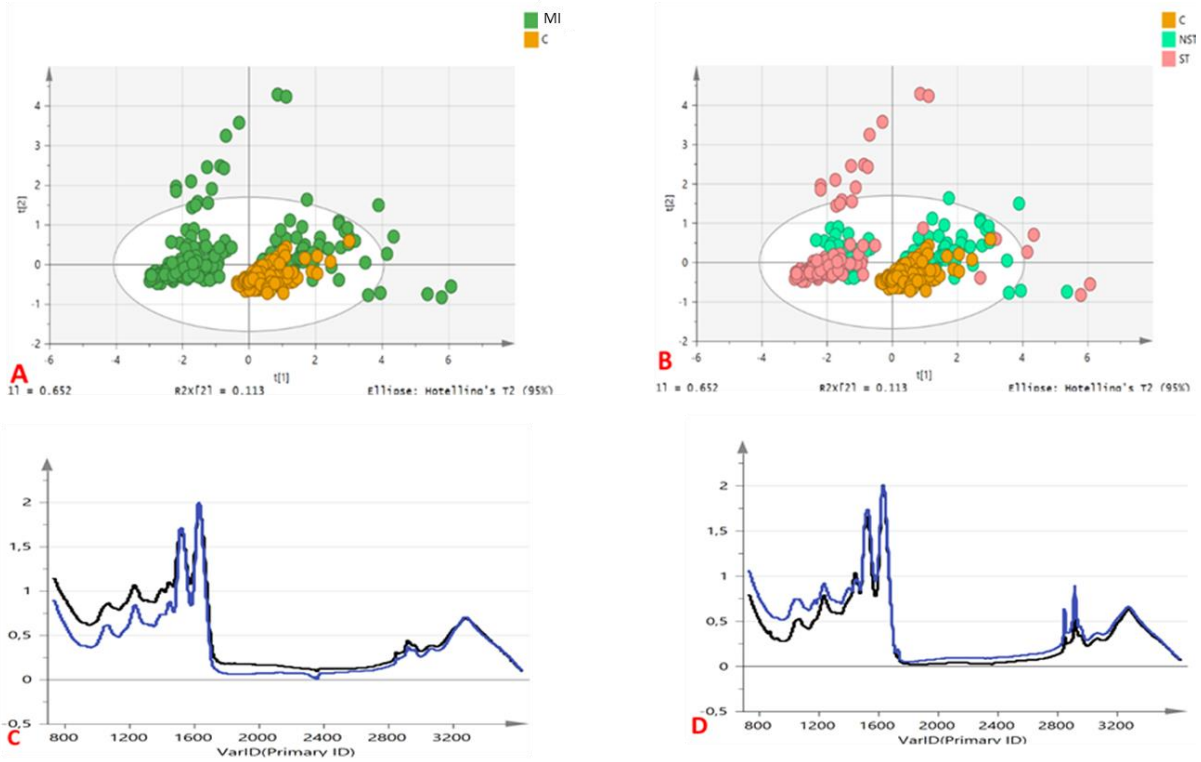


Figure 15: (A) PCA model coloured according to the two groups: acute MI (green) and control (orange). The control group displayed a better relationship as compared to the acute MI group. **(B)**: PCA model coloured according to the three groups; STEMI (pink), NSTEMI (blue) and control (orange). Most NSTEMIs clustered next to controls and only few STEMIs overlapped with the 2 groups (STEMIs = pink, NSTEMIs = blue, and controls = orange). **(C)**: The XObs plot showing where the spectra have similarities and differences between separated STEMIs. Blue line is the randomly selected spectrum from the group overlapping with controls and black line is the randomly selected spectrum taken from the group separating from the controls. **(D)**: The XObs plot showing where the spectra have similarities and differences between separated NSTEMIs. Black line is the randomly selected spectrum from the group overlapping with controls and black line is the randomly selected spectrum taken from the group clustering with STEMIs.

Filters (first derivative) were applied to the spectral data to remove unwanted variations, imperfections in instrument setups and to see if the prediction errors became smaller. These factors contribute to the actual signal and as a result they complicate the creation of good calibration. The model showed better separation (clustering) between the acute MI and control group. Also, there were great variations within the acute MI group with few individuals pulling further away from the clustered group (Figure 16A). Six PCA components were calculated, 58.0% of the variations in the spectral data were explained of which 43.0% were explained in the first three PCA components, this was better than the unfiltered data where 65.2% were explained by the first PCA component and in this PCA model the first PCA component only explained about 19.1% (R2X[1]) of the variation. The filtering removed the noise/unwanted information from the spectra (First PCA component explained lesser fraction when compared with the unfiltered data). The model appears visually better after filtering. When the same model was coloured according to the three groups: the STEMIs appeared to be mostly contributing to the variations within the acute MI group (Figure 16B).

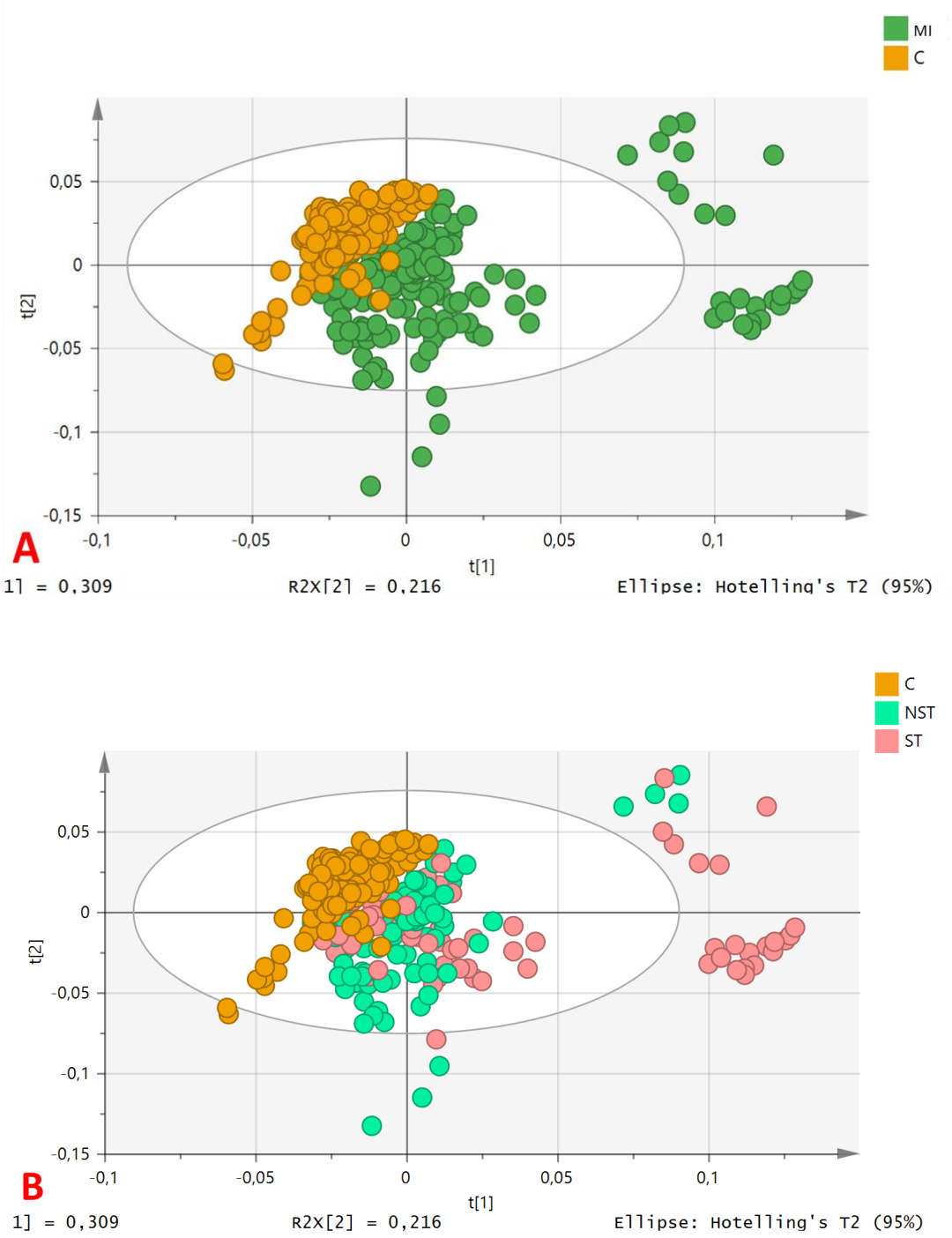


Figure 16: (A) PCA model of the filtered data by first derivative displaying clustering between controls and some partial clustering between acute MI group. The control group displayed a better relationship (clustering) as compared to the acute MI group. **(B)**: PCA model when the observations are coloured according the three groups; STEMIs, NSTEMIs and controls. Some STEMIs separated from the clustered group with few NSTEMIs going with the separated group.

4.3 Discriminant analysis of ATR-FTIR hair spectra from patients with acute MI and healthy controls (females with dyed hair)

Principal Component Analysis (PCA) primary task was to provide an overview of spectral data between the two groups (acute MI and healthy controls), OPLS-DA model was used for further and better separation/discrimination. Orthogonal Projections to Latent Squares Discriminant Analysis (OPLS-DA) separates classes based on the information in the response matrix Y, strong systematic variation in the descriptor matrix X (spectral data) that is orthogonal to Y matrix (80), making it possible to observe the between group and within group variation. The between group variation is observed along the X-direction and the within group variation on the orthogonal direction. In this study, OPLS-DA model separated hair spectral data based on Y components (i.e., chemistry: immunoassays cortisol levels). It separates the predictive from non-predictive (orthogonal) variations (80).

With the introduction of OPLS-DA there was a clear separation between acute MI group and healthy controls. The between group variations from the two groups were seen by the separation along the X-direction. This model was a continuation of the model where 3 cm proximal segments of females with dyed hair from both groups were compared. In Figure 15A it was fitted as a PCA model and in this section, it was fitted as OPLS-DA model (Figure 17A). Most controls are clustered around the X-cartesian plane that separates the first and fourth quadrant; and most cases clustered in the second and third quadrant with a fair share of overlap between the quadrants (second and third).

Although there was a clear separation between the two groups, the clustering was not that good within the acute MI cases. This could be due to acute MI group consisting of two different groups (STEMIs and NSTEMIs) as was the case with PCA model. The predictive component (P1) explained 11.7% of the variation. The model consisted of 10 OPLS-DA orthogonal components, 80.6% of the variation in the spectral data were explained of which 52.7% were explained in the first 2 OPLS-DA orthogonal components. The overall predictive ability, Q2 (cum) was good, 86.6%, it was explained by 90.9% (R2(cum)) of the spectra variation, (Q2/R2 = 95.27%). Corresponding loading plots displayed the peak at 1120 cm^{-1} which corresponds to the cortisol levels region (1102-1180 cm^{-1}) contributes to the variations between the two groups (Figure 17B).

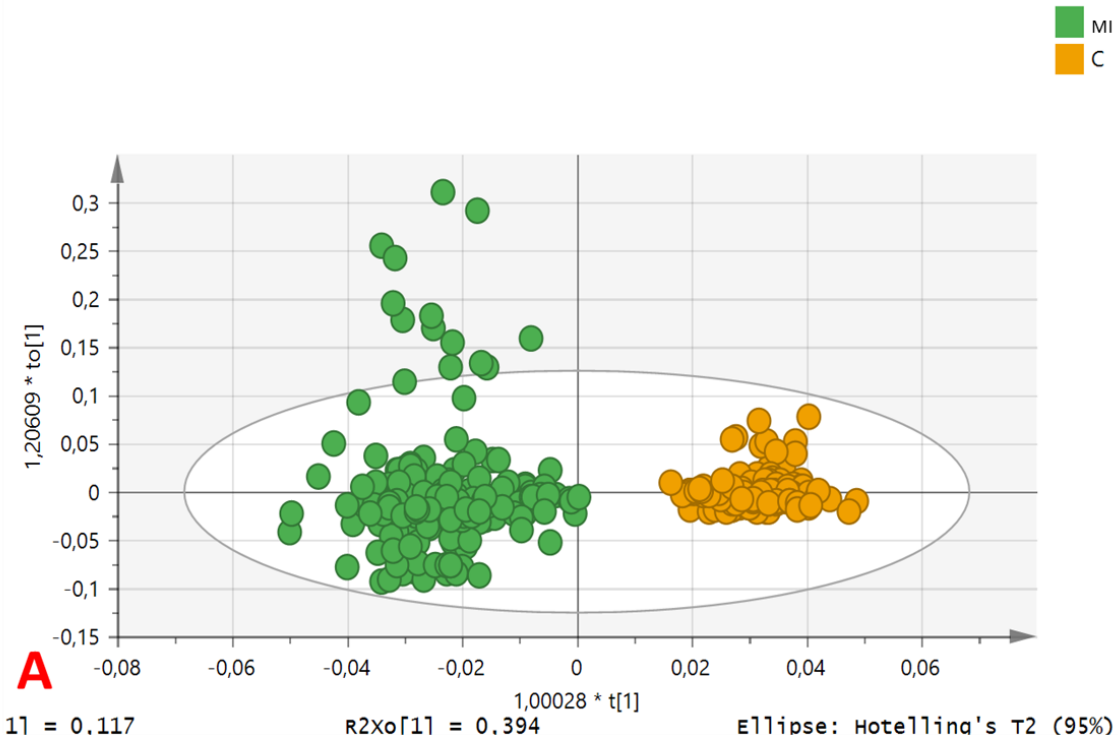


Figure 17: **(A)**: OPLS-DA model displaying a clear separation between acute MI group (green) and healthy controls (orange). **(B)**: The corresponding loading plot displaying the spectra the similarities (blue and green) and differences (Red shows great variations) in the two groups (acute MI and controls). The orange (top end) visualizes the NMR shifts that influence the separation of the groups. The arrow corresponds to the cortisol level region (1102 – 1180 cm^{-1}).

There was also a clear separation between the three groups (STEMIs, NSTEMIs and controls). The STEMIs seems to be the extreme group as they cluster further away from the other two groups. The NSTEMIs are intermediate group since they cluster between both controls and the STEMIs (Figure 18A). The predictive components, P1 and P2, indicated that there were 14.3% and 5.13% of the variations that could be explained. The model consisted of four OPLS-DA orthogonal components, 32.9% of the variations in the spectral data was explained of which 21.1% was explained in the first two OPLS-DA orthogonal components. The overall predictive ability, Q2 (cum) was good, 89.7% and it was explained by 92.1% [R2(cum)] of the spectra variations, (Q2/R2 = 97.39%).

When the two cases from acute MI group (STEMIs vs. NSTEMIs) were compared, there was a clear separation between the groups. The groups are separated by the Y-cartesian plane with all the STEMIs in the second and third quadrant (most in the third quadrant just below the X-cartesian plane) and NSTEMIs on the first and fourth quadrant (Figure 18B). The predictive component indicated that there were 5.24% of the variations that could be explained. The model consisted of five OPLS-DA components, 81.8% of the variations in the spectral data were explained of which 65.5% were explained by the first two OPLS-DA orthogonal components. The overall predictive ability, Q2 (cum) was good, 90.7%, it was explained by 92.5% [R2(cum)] of the spectra variations, (Q2/R2 = 98.05%). The corresponding loading plots between STEMIs and NSTEMIs displayed less variations (Figure 18C) as compared to the one where acute MI group was compared to the controls (Figure 17B).

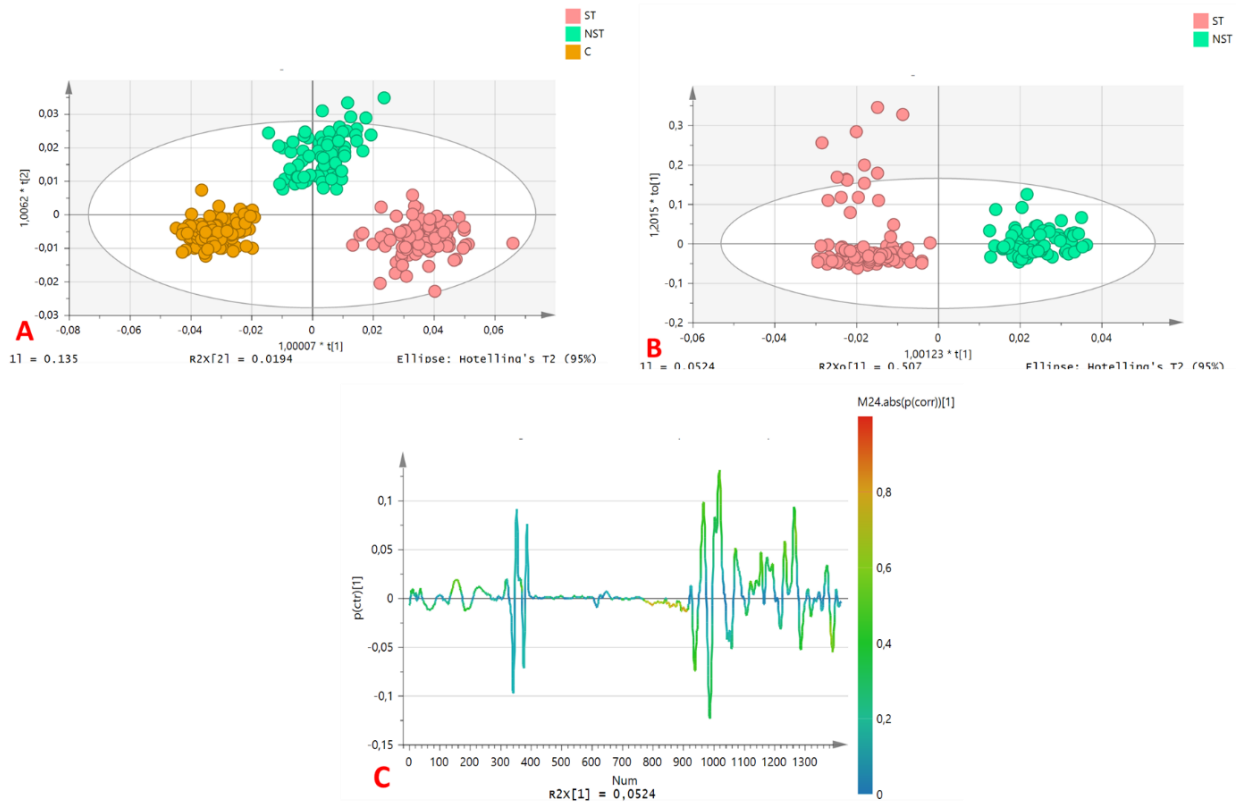


Figure 18: (A) OPLS-DA model showing relationships (clustering) and discrimination (separation) between the two acute MI groups; STEMIs and NSTEMIs, and controls. **(B)** OPLS-DA model when the STEMIs (Pink) are compared with the NSTEMIs (Blue). **(C)** The corresponding loading plot displaying the spectral similarities (blue and green) and differences (Red - shows larger variations) in the two groups (STEMIs and NSTEMIs).

Lastly, when the two acute MI groups; STEMIs and NSTEMIs were each compared to controls, there was a clear separation between the control group and the two acute MI groups. ST-elevation myocardial infarctions (STEMIs) were well separated from the controls (Figure 19A), one participant pulled further away from the rest of the group which created the within group variation. Corresponding loading plots displayed the peak at 1120 cm^{-1} which corresponds to the cortisol levels region (1102-1180 cm^{-1}) contributing to the variations between STEMIs and healthy controls (Figure 19B). The predictive component indicated that there was 15.4% of

the variations that could be explained. The model consisted of five OPLS-DA orthogonal components, 72.4% of the variations in the spectral data were explained of which 43.3% were explained in the first OPLS-DA orthogonal components. The overall predictive ability, Q2 (cum) was good, 93.8%, it was explained by 95.0% [R2(cum)] of the spectra variations. There was also a clear separation between the NSTEMIs and controls (Figure 19C). The predictive component indicated that there was 14.1% of the variation that could be explained. The model consisted of nine OPLS-DA orthogonal components, 75.9% of the variation in the spectral data were explained of which 47.7% were explained in the first three OPLS-DA orthogonal components. The overall predictive ability, Q2 (cum) was good, 91.2%, it was explained by 95.1% [R2(cum)] of the spectra variation. Corresponding loading plots displayed the peak at 1120 cm^{-1} which corresponds to the cortisol levels region (1102-1180 cm^{-1}) contributing to the variations between NSTEMIs and healthy controls (Figure 19D).

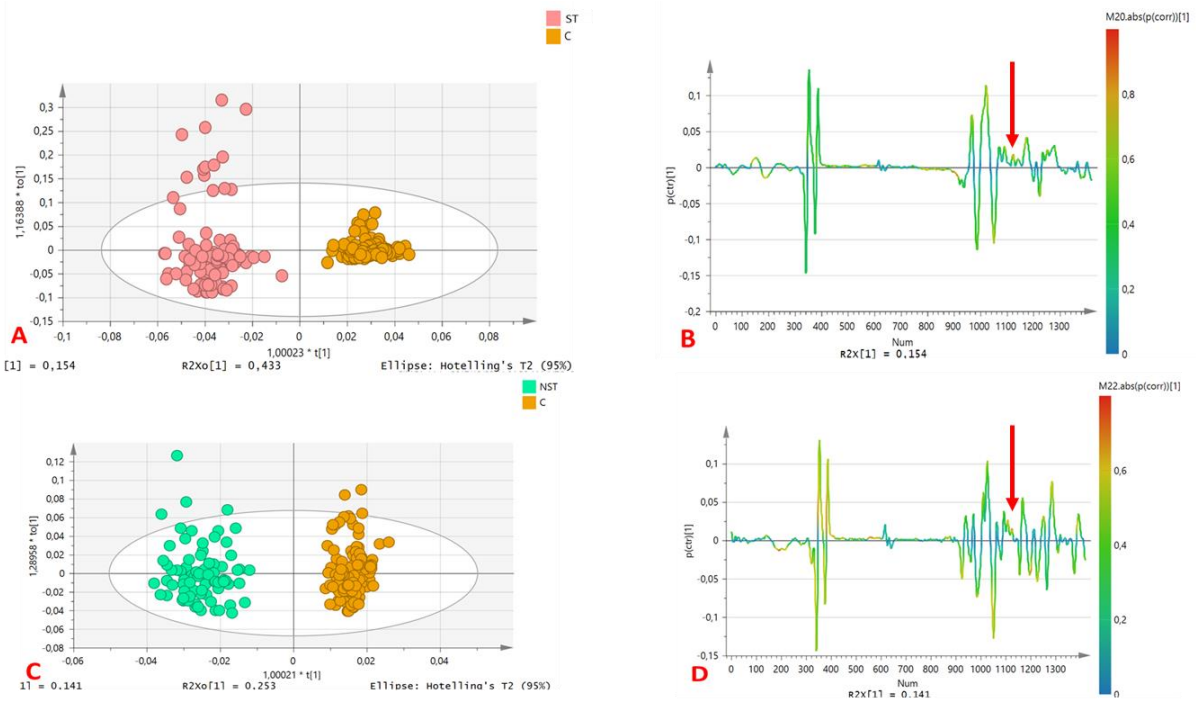


Figure 19: (A): OPLS-DA model obtained from modelling STEMIs (Pink) and controls (Orange). **(B):** The corresponding loading plot displaying the spectral similarities (blue and green) and differences (Red shows great variations) in the two groups (STEMIs and Controls). The arrow corresponds to the cortisol level region (1102 – 1180 cm^{-1}). **(C)**: OPLS-DA model of NSTEMIs (Blue) and controls (Orange). **(D)**: The corresponding loading plot displaying the spectral similarities (blue and green) and differences (Red shows great variations) in the two groups (NSTEMIs and Controls). The arrow corresponds to the cortisol level region (1102 – 1180 cm^{-1}).

4.4 Discriminant analysis of ATR-FTIR hair spectra from patients with acute MI and healthy controls (Natural hair = both male and female cases included)

All controls had dyed hair and as a result the Principal Component Analysis (PCA) and Orthogonal Projection to Latent Structures Discriminant Analysis (OPLS-DA) were only fitted on dyed hair in the earlier analysis. In this section since, it was just a comparison of acute MI cases with natural hair. When STEMIs and NSTEMIs with proximal segments of natural hair were compared (STEMIs and NSTEMIs), there was a partial separation between the two groups, they are separated by the Y-cartesian plane with almost all the STEMIs on the second and third quadrant, and NSTEMIs on the first and fourth quadrant (Figure 20A). Corresponding loading plots displayed no peak difference corresponding to cortisol region (1102-1180 cm^{-1}) between STEMIs and NSTEMIs (Figure 20B). The predictive component indicated that there was only 29.8% of the variation that could be explained. The model consisted of 13 OPLS-DA orthogonal components, 89.9% of the variations in the spectral data were explained of which 28.2% were explained in the first OPLS-DA orthogonal component. The overall predictive ability, Q2 (cum) was good, 70.5%, it was explained by 78.5% [R2(cum)] of the spectra variation, (Q2/R2 = 89.81%). When the model was fitted with distal segments of natural hair, the model collapsed, which suggests that there was little or no variations between the two (Figure 20C). Corresponding loading plots displayed more variations and there was also a peak at 1120 cm^{-1} which corresponds to the cortisol levels region (1102-1180 cm^{-1}) (Figure 20D). The predictive component indicated that there were only 33.1% of the variations that could be explained. The overall predictive ability, Q2 (cum) was not good, 31.5%, it was explained by 32.2% [R2(cum)] of the spectra variation.

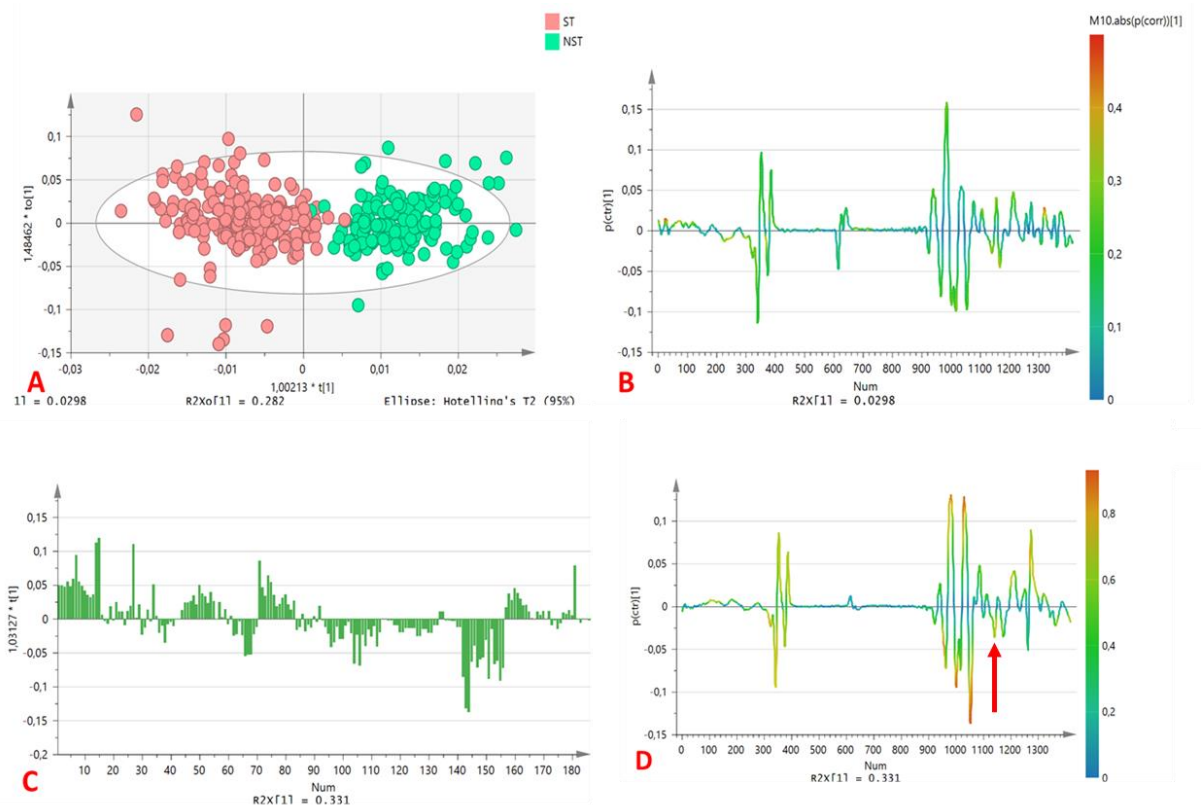


Figure 20: **(A)** OPLS-DA model when STEMIs (Pink) are compared with NSTEMIs (Blue) of proximal segments of natural hair (12 STEMIs and 8 NSTEMIs). **(B)** The corresponding loading plot displaying the spectral data similarities (Blue and green) and differences (Red) of the natural proximal segments between STEMIs and NSTEMIs. **(C)** OPLS-DA model when STEMIs are compared to NSTEMIs for distal segments of natural hair (7 STEMIs and 4 NSTEMIs). **(D)** The corresponding loading plot displaying the spectral similarities (blue and green) and differences (Red shows great variations) of the natural distal ends between STEMIs and NSTEMI. The arrow corresponds to the cortisol level region (1102 – 1180 cm^{-1}).

4.4.1 OPLS-DA model validation

Permutation plots were used to validate each OPLS DA model, all the models were valid since they all had y intercept values of below 0 (Q2s), and R2 values were all below 0.30 (Figure 21). The permutation models is an easy way and efficient way of checking how far a model is from being a coincidence correlation by performance; it helps in assessing the validity of a regression model (86). It is a sort of internal validation scheme, in cases where the significance of the model is doubtful it provides crucial information on whether to keep or reject the model (86). Permutation plots make sure that models are not spurious and overfit. They also ensure that if a new sample was to be introduced into the model, it would be able to accurately predict which group the sample belongs to.

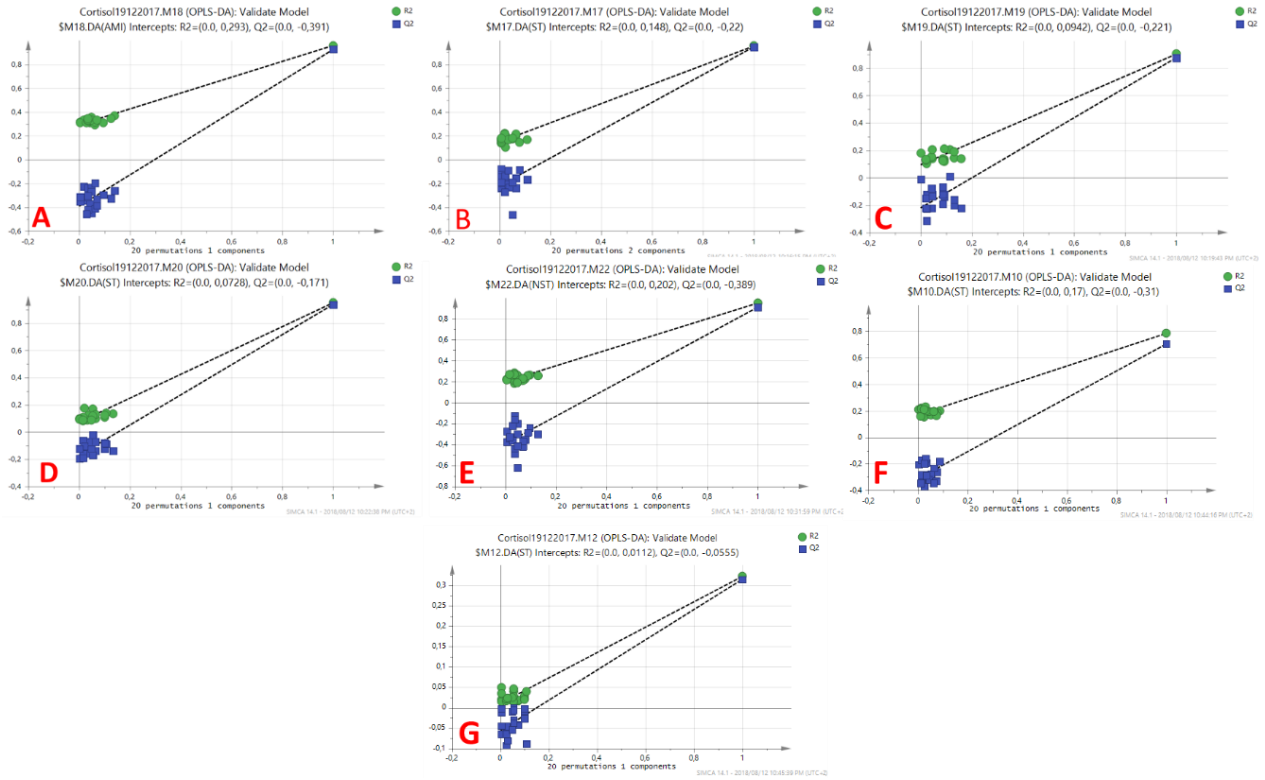


Figure 21: Permutation plots for the internal validation of OPLS-DA models for acute MI vs. controls with dyed hair **(A)**, STEMIs, NSTEMIs and controls with dyed hair **(B)**, STEMIs vs. NSTEMIs with dyed hair **(C)**, STEMIs vs. controls with dyed hair **(D)**, NSTEMIs vs. controls with dyed hair, **(E)** STEMIs vs. NSTEMIs with natural hair (Proximal segments) **(F)** and **(G)** STEMIs vs NSTEMIs with natural hair (Distal segments).

4.4.2 Classification tables for each of the OPLS-DA models

Classification tables indicate how well the model can classify hair spectra based on assigned groups. The OPLS-DA models were able to predict spectra according to specified groups with 98.68-100% accuracy for proximal hair segments and 50-94.62% for distal hair segments where the model failed to separate STEMIs from NSTEMIs (Tables 3 - 9). The “members” represents the number of points on hair strands (5 per hair strand) not the actual number of participants.

Table 3: Classification table for spectra obtained from dyed hair of acute MIs vs. healthy controls.

	Members	Correct	MI	C	No class (YPred <= 0)
MI	196	100%	196	0	0
C	132	100%	0	132	0
No class	0		0	0	0
Total	328	100%	196	132	0
Fisher's prob.	0				

Table 4: Classification table for spectra obtained from dyed hair of STEMIs, NSTEMIS and healthy controls.

	Members	Correct	ST	NST	C	No class (YPred <= 0)
ST	104	100%	104	0	0	0
NST	79	100%	0	79	0	0
C	131	100%	0	0	131	0
No class	0		0	0	0	0
Total	314	100%	104	79	131	0

Table 5: Classification table for spectra obtained from dyed hair of STEMIs vs. NSTEMIs.

	Members	Correct	ST	NST	No class (YPred <= 0)
ST	102	100%	102	0	0
NST	78	100%	0	78	0
No class	0		0	0	0
Total	180	100%	102	78	0
Fisher's prob.	0				

Table 6*:* Classification table for spectra obtained from dyed hair of STEMIs vs. healthy controls.

	Members	Correct	ST	C	No class (YPred <= 0)
ST	104	100%	104	0	0
C	132	100%	0	132	0
No class	0		0	0	0
Total	236	100%	104	132	0
Fisher's prob.	0				

Table 7: Classification table for spectra obtained from dyed hair of NSTEMIs vs. healthy controls.

	Members	Correct	NST	C	No class (YPred <= 0)
NST	79	100%	79	0	0
C	131	100%	0	131	0
No class	0		0	0	0
Total	210	100%	79	131	0
Fisher's prob.	0				

Table 8: Classification table for spectra obtained from proximal hair of STEMIs and NSTEMIs with natural hair.

	Members	Correct	ST	NST	No class (YPred <= 0)
ST	255	99,22%	253	2	0
NST	151	98,68%	2	149	0
No class	0		0	0	0
Total	406	99,01%	255	151	0
Fisher's prob.	0				

Table 9: Classification table for spectra obtained from distal hair of STEMIs and NSTEMIs with natural hair.

	Members	Correct	ST	NST	No class (YPred <= 0)
ST	130	94,62%	123	7	0
NST	56	50%	28	28	0
No class	0		0	0	0
Total	186	81,18%	151	35	0
Fisher's prob.	9,90E-12				

4.5 Investigating the correlation between hair cortisol concentration and ATR-FTIR spectra using partial least squares (PLS) regression

Partial Least Squares (PLS) is based on the principle of assessing relationships between a descriptor matrix X and a response Y (80). Since PLS explains overall class properties (STEMIs, NSTEMIs and healthy controls) which had variable cortisol levels, the interpretation was not that good for the cases where the three groups were included, this could also be affected by the longer-range differences (1.43-209.18 pg/mg) between the groups (Figure 22). This resulted in the model showing negative values, which is not true since all the Immunoassay cortisol results were all above 1.00 pg/mg. The model consists of five PLS components which used 99.9% (R2X (cum)) of the variation of the spectral data to explain 74.3% (R2Y(cum) of the variation obtained from Immunoassay cortisol concentration. The predictive ability of the models was good, Q2(cum), 72.5%. The model had a good correlation coefficient (R^2 = 0.8488).

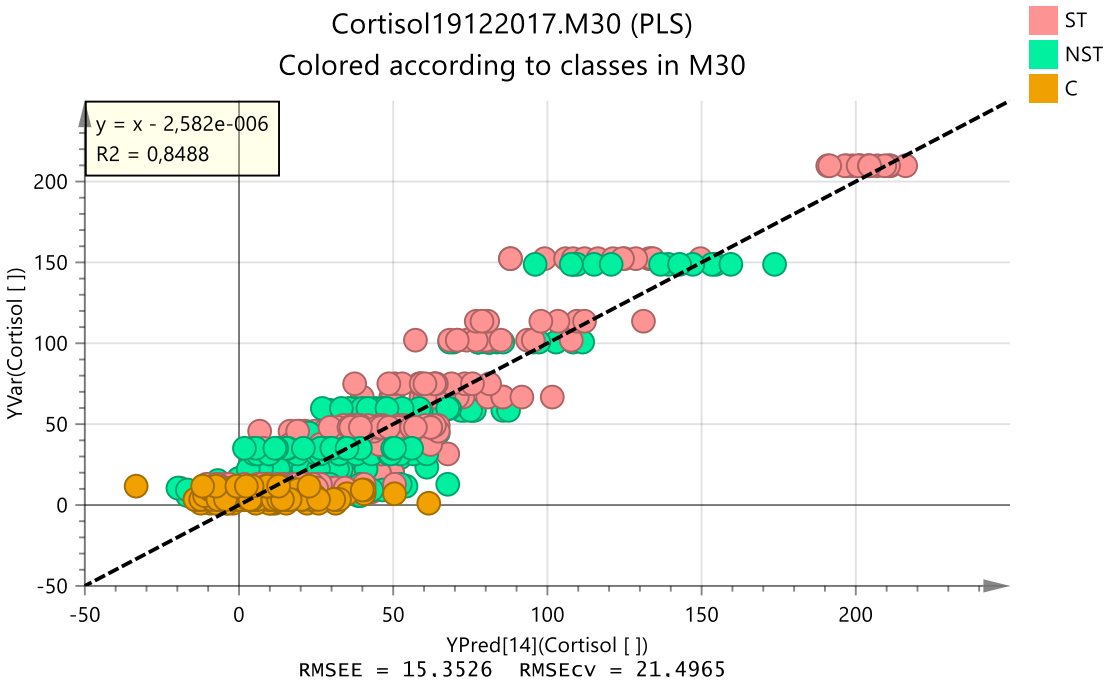


Figure 22: Displaying the overall PLS model between the three groups; STEMIs (Pink), NSTEMIs (Blue), and Controls (Orange).

4.5.1 PLS model validation

A permutation plot was used to validate the PLS model. PLS permutation plots indicates whether a PLS model is valid and not overfit i.e., it determines whether the PLS model would be able to predict hair cortisol concentrations levels for new spectra. The model was valid since the intercept value for Q2 was below 0 (-0.28), and R2 value was below 0.3 (0.191) (Figure 23).

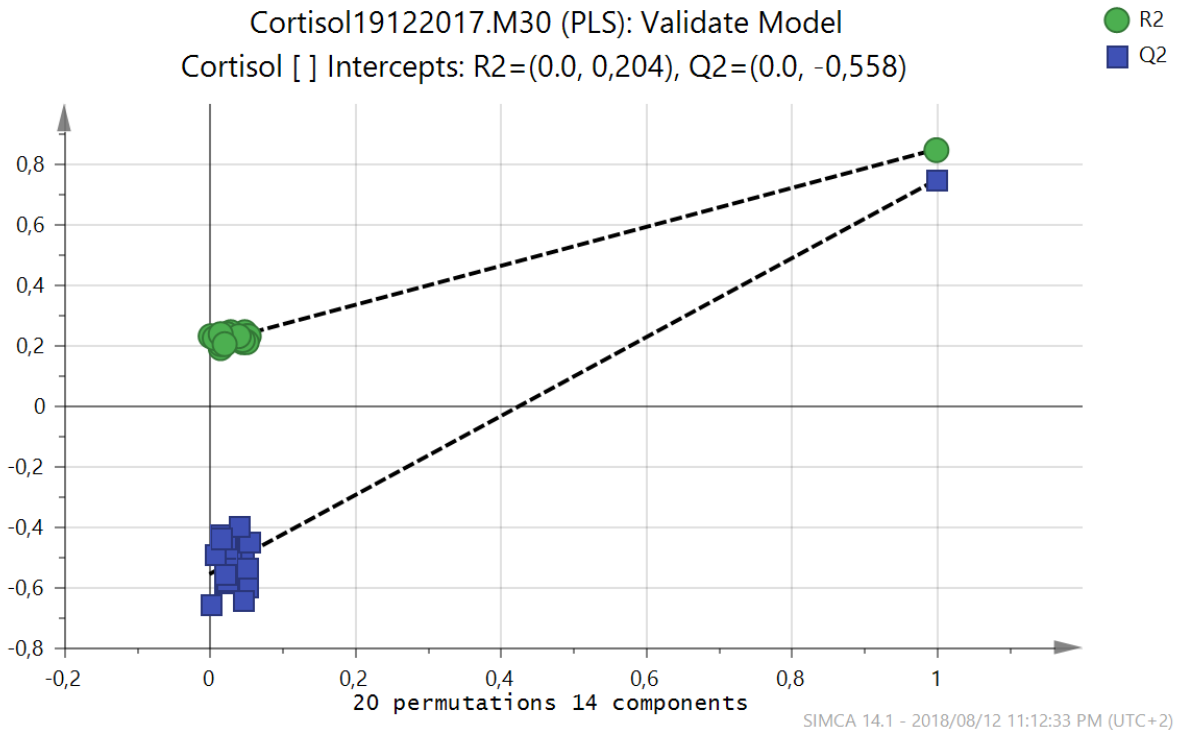


Figure 23: Permutation plot of the overall PLS model between the three groups: STEMIs, NSTEMIs and controls.

CHAPTER 5: GENERAL DISCUSSION

The aim of the study was to investigate the use of hair cortisol as a biomarker of chronic or pre-existing stress in patients who had sustained a recent acute myocardial infarction (MI). To the authors' knowledge, this is the first study that used both enzyme-linked immunoassays (ELISAs) and attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy to examine whether there were any differences between the two types of acute MI and healthy controls. This involved comparing hair cortisol levels between the two types of acute MIs; ST-elevation myocardial infarction (STEMIs) and non-ST-elevation myocardial infarction (NSTEMIs) and healthy controls. The hair was also subjected to ATR-FTIR spectroscopy and multivariate data analysis to detect differences in the hair chemical profiles between the groups. Finally, data was tested for any correlation between hair cortisol levels and ATR-FTIR spectroscopy spectral data. Immunoassays and multivariate analysis are both sensitive techniques, therefore it was important to record participants information like hair treatments, gender, age, etc., to reduce variations among participants more especially when doing comparison of groups using SIMCA software; the groups had to be of similar features.

5.1 Optimization of cortisol extraction

One of the main challenges during this project was collecting enough hair during the sampling process. While study participants easily volunteered for hair samples due to the non-invasive nature of the collection process, they were reluctant to have too much hair taken. Therefore, hair was sampled conservatively so not to upset study participants but we aimed for a minimum of 100 strands. Different studies have used different extraction methods for Immunoassays analysis i.e., washing procedures, hair weights, amount of methanol, etc. (15,

81, 87-92). Hair was weighed after sampling and as little as 50 mg of hair was sampled. Although, the amount of hair was expected to be one of the limiting factors in extracting and measuring cortisol levels in hair this proved not to be a fact. Study optimizations were first done to evaluate the amount of hair and dilutions that worked best for the immunoassay kit used. The optimization results showed that 10 mg of hair and the 1:20 dilution worked best for cases and 10 mg and undiluted worked best for controls. This method was then adopted for the rest of the study. The cases immunoassay cortisol levels from optimizations were more than 20-fold than that of healthy controls.

5.2 Comparison of cortisol levels in patients with MI and healthy controls

In long hair, cortisol levels are reported to represent long-term/chronic stress since hair grows at an average of a centimetre a month and the length can be correlated to preceding months (18). This enabled a long-term analysis/monitoring of the *hypothalamic-pituitary-adrenal* (HPA)-axis. Depending on hair length cortisol levels could be used to examine stress levels at least 9 and 3 months before as well as 3 months after the heart attack. Since 3 cm of hair were used all results were reflecting chronic stress as acute stress only lasts up to 4 weeks (93).

This study found total elevated hair cortisol levels in acute MI cases compared to healthy controls, indicating increased cumulative cortisol levels in the preceding 9 months before the heart attack. This is consistent with data reported by Pereg et al., who found elevated hair cortisol levels among acute MI cases compared to controls. These authors suggested that

chronic stress (accompanied by elevated cortisol levels) might be a contributing factor to acute MI (11). Another study by Dettenborn et al., reported elevated cortisol levels among unemployed individuals compared to controls. The authors found significant difference in the first segment (proximal 3 cm from the scalp - corresponding with 3 months) and second segment (3-6 cm from the scalp - 6 months), but there was no significant on the third segment. The authors suggested that this reduction in cortisol levels (segment 3 vs. segment 1 and 2) may be a result of repeated washing of hair (i.e., the wash-out effect) (70). The unemployed individuals stress questionnaires indicated increased levels of perceived stress and impairments in subjective well-being compared to employed individuals, but they found no relation between these data and cortisol levels in 3 cm hair segments (70). Contrary to this, Karla et al., reported positive correlation between validated questionnaires (that detect 30 days ~ about 4weeks) of perceived stress with elevated cortisol levels in 1.5 cm segments (6 weeks) of hair in pregnant women (17). The reason why there was no correlation in the unemployed participant study was likely that the longer duration reflected by 3 cm hair segments did not match the shorter time period reflected by stress questionnaires (94). To our knowledge this is the first study that investigated the difference in cortisol levels between the two types of acute MI (STEMIs and NSTEMIs). The total cortisol levels showed significant difference between STEMIs, NSTEMIs and controls.

To take into account the issue of wash-out effect, which suggests that hair of greater than 6 cm may have inconsistently low cortisol levels (70, 91), the acute MI group and the control group distal segments (9-12 cm) were also compared amongst each other. Although there might be a wash-out effect in hair greater than 6 cm, this study still did not find evidence of this effect, i.e., the cortisol levels in acute MI cases were significantly higher compared to controls (94% of controls had very low cortisol levels, i.e., below 10 pg/mg). The 3 cm hair proximal cortisol levels were higher in acute MI cases than healthy controls. There was a significant difference in terms of total cortisol levels (proximal and distal) between STEMIs, NSTEMIs and controls. However, there was no significant difference when STEMIs and NSTEMIs hair cortisol from proximal segments were compared (both groups showed substantially higher levels than controls). There was also no significant difference when STEMIs and NSTEMIs distal segments were compared, but they were both still significant when compared to controls. The distal segments result between the acute MI cases were expected to be significantly different since the total cortisol levels showed a significant difference and the proximal segments result also showed no significant difference; although there was no significant difference in distal segments cortisol levels between STEMIs and NSTEMIs ($p>0.05$). The p-values for both proximal and distal segments of acute MI cases showed no significant difference, it needs to be noted that the p-values were very different, 0.257 and 0.051, proximal vs. distal ends, respectively (i.e., the difference in the distal segments just narrowly missed the cut-off level of significance).

The high variability of p-values between proximal and distal segments cortisol levels of acute MI cases could be due to the following factors; 1. cortisol levels might have been at the initial

stage of accumulation in the distal hair segments of NSTEMIs, 2. There was a wash-out effect that does not have any significant difference in the within group variation (i.e., when the individual's proximal and distal cortisol levels were compared). These results suggest that acute MI cases were suffering from chronic stress for prolonged periods (9 months or more especially in the case of STEMIs - higher cortisol levels). The STEMIs had elevated cortisol levels which not only predated the acute MI but were unchanged throughout the hair segments (i.e., periods of 9 months or more). Interestingly, elevated cortisol levels also predated acute MI but more than doubled in the proximal segments preceding 3 months (correlating with 3 months) before the heart attack in NSTEMIs, [29.64 (5.87-148.420 vs. 9.94 (5.15-32.95) pg/mg].

To further investigate cumulative cortisol levels differences between the two acute MI types, proximal and distal segments within the same individual were compared (Figure 12). The high cortisol levels in proximal and distal segments were not statistically different in STEMIs, ($p>0.05$). Total cortisol levels in STEMIs were higher than in NSTEMIs (both were higher than controls), [35.18 (8.15-209.18) vs. 17.24 (3.73-148.22) pg/mg], ($p<0.001$), in STEMIs vs. NSTEMIs, respectively, and in controls they were [3.32 (0.37-11.24) pg/mg]. Interestingly, proximal hair segment cortisol levels (3 months before the heart attack) were significantly higher than distal segment in NSTEMIs, ($p<0.01$). These findings are consistent with the total cortisol analysis (proximal and distal combined) which suggest that STEMIs may be subjected to chronic stress over the 9 months period prior the heart attack. For NSTEMIs the results suggest a recent increase in cortisol levels over the past 9 months whereas STEMIs have been

chronically constantly stressed for prolonged periods (the fact that distal ends for NSTEMIs are still higher than controls).

One plausible reason why STEMIs maybe suffering from chronic stress more than NSTEMIs could be due STEMIs consistently having stressful jobs or family issues whereas NSTEMIs could be experiencing a sequence of recent stressful events that lead to a sudden rise in stress levels. There was no significant difference in cortisol levels between proximal and distal segments of healthy controls. This suggest that controls did not suffer from any chronic stress for a period of nine months or more. These findings firmly support the Tirosh et al., study which suggested that the higher cortisol levels among the Cushing Disease (CD) patients may explain the differences in the coagulation profile as well as the higher risk for venous thrombo-embolic events (VTEs) (95). Furthermore, the extent of coronary disease in the cases studied has not been clearly defined. It is likely that STEMIs had more advanced coronary disease than NSTEMIs and had more severe coronary events/symptoms in the preceding nine months which might have contributed to their higher cortisol levels.

It is conceivable that acute MI patients suffer from acute physical stress. Although the numbers were small the comparison of STEMIs before and after proximal segments showed a significant decrease in cortisol levels after the heart attack. Cortisol levels rise in response to any type of stress: psychological or physical and decline once the stressful event has passed/or has been treated. This may further support the earlier suggestion that the higher prevalence of VTEs in patients with CD may be due to high cortisol levels associated with the

disease. This was expected as the patients received treatment (i.e., anti-coagulants like enoxaparin, dalteparin, fondaparinux) which should be reducing the size of the blockage in STEMI patients. Other treatments include: antiplatelets (asprin, clopidogrel, ticagrelor), statins (atorvastatin, rosuvastatin, simvastatin, pitavastatin), Angiotensin II Receptor Blockers (ARBs - valsartan, candesartan, losartan, olmesartan), angiotensin-converting-enzyme inhibitor (ACE inhibitor - ramipril, enalapril, captopril, lisinopril) (32), to name a few. Therefore, treatment might have influenced the reduction in post MI cortisol levels. It would have been interesting to have more NSTEMI patients after heart attack to determine whether the cortisol levels were still increasing significantly or not.

5.3 Comparison of ATR-FTIR hair spectra from MI patients and healthy controls

The results of spectral data analysis supported hair cortisol measurement by immunoassays. The data showed that there was a difference between acute MI cases and healthy controls (Figure 17A). Acute myocardial infarction (MI) cases suggested a greater within group variations. When this was further investigated, it was found that the variations were due to the acute MI group having two different groups (STEMIs and NSTEMIs). Most of the individuals that were responsible for the within group variations were STEMIs. However, this might not be the only factor contributing to the variations, it could be a combination of factors as people experience different stress levels due to having different lifestyles. Many of the acute MI cases were either diabetic or had high blood pressure or both in some cases. It is difficult to exclude such confounders as they all form part of the acute MI risk factors. When

STEMIs were compared to the NSTEMIs age was identified as a possible confounder as one patient that was responsible for the variation in the STEMI group was the youngest in the group (aged 33), indicating age as a potential confounder. The within group variation could be due to dehydroepiandrosterone (DHEA) as it is age dependent and may account for the separation and variation on multivariate analysis. Circulating DHEA levels are lower in first years of life and increase between age 6-10, and the levels are at a maximum from the third decade, followed by the steady decline with increasing age (96, 97).

Multivariate data analysis results were also consistent with immunoassay results that suggested that STEMIs had higher cortisol levels as the OPLS-DA model showed that controls clustered closer to the NSTEMIs (with STEMIs further away from the controls) (Figure 18A). Although, it needs to be noted, unlike in immunoassay results above, these results were not only based on cortisol levels (they were based on whole hair spectra). Multivariate data analysis was used to determine whether the results were in line with immunoassay results and if possible do further analysis were immunoassays analysis fell short. When both natural and dyed hair proximal segments between STEMIs and NSTEMIs were compared there was a partial separation between the two. The model collapsed when the natural distal segments between STEMIs and NSTEMIs were compared. Interestingly, the corresponding loading plot for proximal segments for natural hair displayed less variations in the cortisol region (1102-1180 cm) when compared to the one for distal segments (Figure 20B vs. Figure 20D). This is consistent with immunoassays level of significance that revealed that the distal segments were not statistically significant, but they just missed the cut-off level [proximal (p-value = 0.257) vs. distal (p-value = 0.051)]. Therefore, in terms of cortisol levels according to the

corresponding loading plots there is a difference between STEMIs and NSTEMIs. Thus, multivariate data analysis could also provide further analysis for validations and even for improvements where there was lack of clarity. Furthermore, since OPLS-DA did manage to separate the two acute MI groups, then multivariate data analysis does indeed prove to be a useful tool for further validation of immunoassay analysis.

Although, the separation of STEMIs and NSTEMIs in multivariate data analysis could be due to several chemical profile differences between the two groups, cortisol might also be a contributing factor (higher variability in the cortisol region of corresponding loading plots between STEMIs and NSTEMIs). Cortisol is a complex molecule and if there is a difference in cortisol concentrations in the two, which proved to be the case in this study, accounting for the separation on multivariate analysis. The separation might also be related to an ischemic event (i.e., ST-segment, whether it depressed or elevated - STEMIs vs. NSTEMIs). This study showed that alternative ways to avoid potential confounders, like hair treatments, is to categorize hair, e.g., according to segments (proximal vs. distal ends), chemical treatment (natural vs treated hair), etc.

The OPLS-DA models were used to discriminate between hair spectral data of acute MI group (including the ones where the group was separated into STEMIs and NSTEMIs) and healthy controls. The separation of spectral data for proximal hair segments between groups were excellent. The predictive abilities were also excellent with Q2(cum) ranging from 0.705 to 0.938 and classification abilities ranged from 98.68 to 100%. This demonstrates that it is

possible to detect cortisol and separate the two acute MI groups (STEMIs and NSTEMIs) with the use of ATR-FTIR. This could be a major breakthrough since STEMIs experiences a sudden occlusion to the coronary artery, therefore cortisol and FTIR analysis could be used as the biomarkers for this type of acute MI. However, this requires a larger database of spectra from which calibration sets could be built into ATR-FTIR hand tool devices for detecting cortisol and classifying the risk status of individuals prior MI.

Pereg et al., study found hair cortisol levels to be an independent predictor for acute MI after controlling for independent predictor for the conventional risk factors of CVD, but they also stressed that it could be possible that the abnormalities may contribute to the increased cardiovascular risk associated with chronic stress (11). This study further supports and clarifies this since it was able to separate the two acute MI groups based on cortisol. Our data support hair cortisol analysis (as a measurement of chronic psychosocial stress) as an additional risk factor for MI.

5.4 The correlation between hair cortisol and ATR-FTIR spectra

When immunoassay cortisol concentrations were correlated with FTIR results the data showed a good correlation coefficient, 0.85. The results were also consistent with immunoassay and OPLS-DA which suggested that the ST-elevation myocardial infarctions (STEMIs) tends to fall between the non-ST-elevation myocardial infarctions (NSTEMIs) and healthy controls.

5.5 Limitations of the study

This is a pilot or proof of concept study and results require validation in larger studies. The other limitations were gender and age of cases compared to controls (controls were all females and were younger than cases). Although, the analysis was heavily weighted on females (all controls were females), the study with female-only analysis was conducted excluding all males from the study with all participants. Interestingly, the two studies showed similar results (Table 2 and Figure 13). These findings proved that sexual dimorphism did not play a role in stress responses, infarction and cortisol levels.

The reason why controls were younger than cases was that the author attempted to recruit participants from within the same area. Therefore, controls were recruited as people who were accompanying/visiting patients at the hospital, unfortunately they happened to be younger than cases. The study by Zouaghi et al., reported that there was little, or no difference in mean serum cortisol concentrations between old and young patients (57), therefore age might not be a contributing factor in the differences in cortisol levels in this study. Unlike dehydroepiandrosterone (DHEA), age is a plausible confounder as DHEA varies with age. The sample size where the within group cortisol levels (proximal versus distal) in ST-elevation myocardial infarctions (STEMIs) might be of a concern as there were only eight participants.

All multivariate analysis models were validated internally through permutation plots. Technically, calibration models are supposed to be created and externally validated by independent test sets of new experiments made up of entirely new participants to ensure classification and biomarkers which caused separation were the same. The sample size was

not adequate to perform such tests, i.e., splitting each group into calibration and test sets without compromising calibration sets outcomes. However, this study did postulate proof of concept that hair cortisol could be used as a biomarker of chronic psychosocial stress in patients with acute MI compared to healthy controls. For future studies, larger sample sizes should be recruited to enable calibration sets of individuals with acute MI.

5.6 Conclusions

This study suggests that chronic constantly high stress (based on hair cortisol levels) predates MI. Although hair cortisol levels were also high in NSTEMIs (but lower than STEMIs) they more than doubled before the onset of an acute MI. This study confirms the usefulness of hair cortisol as a potential tool for the diagnosis of chronic stress. The findings from the combination of the use of immunoassays and ATR-FTIR analysis is a novel addition to existing knowledge that maybe useful in future for clinical research. The ATR-FTIR technique is a cost effective and easy to use and could be used as a better replacement for expensive tests in future. Future studies should evaluate hair cortisol against other risk factors (DHEA, and lipids) and confirm the reliability of using hair cortisol as a marker of chronic stress in cardiovascular diseases. The authors of this study suggest that the cortisol levels may have been higher in STEMIs possibly because they had more advanced coronary disease and were therefore more symptomatic in the preceding months compared to NSTEMIs. The levels in the NSTEMIs doubled near the time of infarction because they had less severe disease with fewer symptoms of angina, and probably became more symptomatic near the time infarction.

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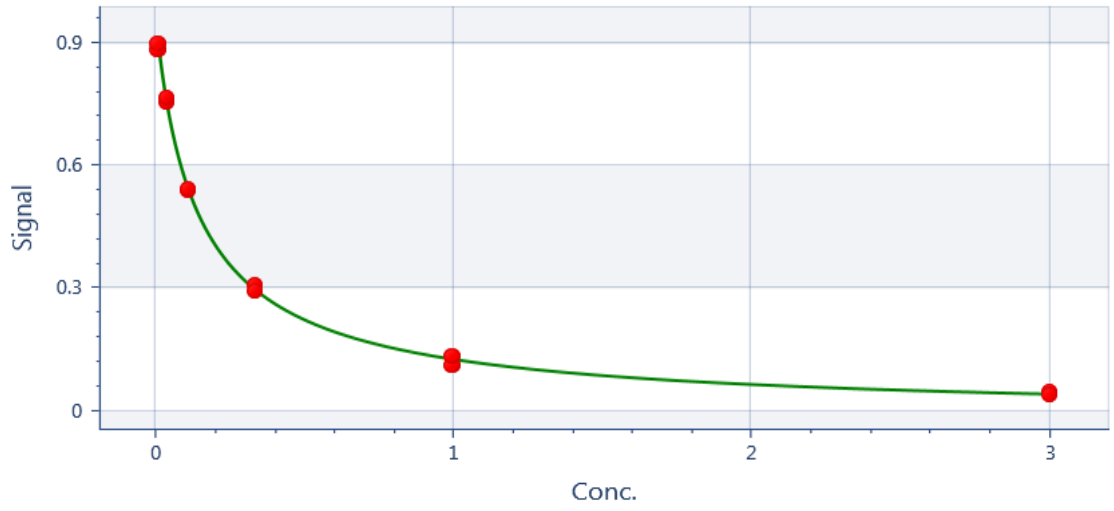
APPENDIX



Appendix 1*:* Standard reference curve.

Appendix 2*: Ethics approval letter of the study*

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6626
Email: shurette.thomas@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

29 September 2016

HREC REF: 451/2016

Prof N Khumalo
Dermatology
G23, New Groote Schuur Hospital

Dear Prof Khumalo

PROJECT TITLE: THE USE OF HAIR CORTISOL AS A BIOMARKER OF STRESS (MSc-candidate-ME Mabotha)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee.

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

Approval is granted for one year until the 30th September 2017.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

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

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval before the research may occur.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH

2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI),and Declaration of Helsinki (2013) guidelines.
The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

Appendix 3: *Project title amendment approval.*

UNIVERSITY OF CAPE TOWN

FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

Form FHS006: Protocol Amendment

HREC office use only (FWA00001637; IRB00001938)		
☑ Approved	☑ Type of review: Expedited	☐ Full committee
This serves as notification that all changes and documentation described below are approved.		
Signature Chairperson of the HREC		Date: 15/02/2018

Note: All major amendments must include a local PI synopsis justifying the changes for the amendment. Please note that incomplete amendment submissions will not be reviewed.

Comments from the HREC to the Principal Investigator:

HUMAN RESEARCH ETHICS COMMITTEE
15 FEB 2018
HEALTH SCIENCES FACULTY
UNIVERSITY OF CAPE TOWN

Note: The approval of this protocol amendment does not grant annual approval. Please complete the FHS016 / FHS017 form for annual approval at least one month before study expiration.

Principal Investigator to complete the following:

1. Protocol Information

Date (when submitting this form)	14/02/2018
HREC REF Number	451/2018
Protocol title	THE USE OF HAIR CORTISOL AS A BIOMARKER OF PSYCHOSOCIAL STRESS IN PATIENTS WITH A RECENT ACUTE MYOCARDIAL INFARCTION
Protocol number (if applicable)	
Principal Investigator	Prof Nonhlanhla Khumalo
Department / Office Internal Mail Address	Medicine mkhumalo2@uct.ac.za

1.1 Is this a major or a minor amendment? (see FHS006hlp) Major (tick box) Minor (tick box)	☐ Major	✓ Minor
1.2 Does this protocol receive US Federal funding?	☐ Yes	✓ No
1.3 If the amendment is a major amendment and receives US Federal Funding, does the amendment require full committee approval? **Note:** Any protocol amendments for **Full Committee** review MUST be submitted on the monthly HREC submission dates.	☐ Yes	☐ No

Appendix 4*: Consent form.*

The use of hair cortisol as a biomarker of psychosocial stress in patients with a recent acute myocardial infarction

Informed Consent form (acute myocardial infarction patients)

Dear Volunteer

Scalp hair is exposed to and incorporates many products that circulate in our blood. We, at the Hair and Skin Research Lab at the University of Cape Town are doing a study to compare hair cortisol levels in patients who have suffered a heart attack versus an age matched healthy control. We further intend to determine whether the lipid (or fat) content in hair influences how much of the normal hormone cortisol is incorporated into human hair. Cortisol is a steroid hormone which regulates a wide range of processes throughout the body including metabolism and the immune response. Cortisol achieves these processes by controlling the body's blood sugar levels and thus regulating metabolism, acting as an anti-inflammatory, influencing memory formation, controlling salt and water balance, and influencing blood pressure. It has a very important role in helping the body respond to stress. Very high levels of cortisol have been shown to correlate with stressful conditions such as a heart attack. Hair length can be used to detect how far back a person was exposed to high cortisol levels unlike blood cortisol which change throughout the day. We would like to compare hair cortisol levels in patient's 3-6 months after a heart attack with that of age-matched healthy controls.

Do I have to take part?
Your participation in the study is optional. However, there are minimal risks in participating as there is no treatment involved in hair sample collection, hence there will be no side effects.

Will I receive payment for taking part? No there is no payment for participating in the study as you will be seen on your normal visit to the clinic.

What will I benefit by participating?
There will be no immediate benefit. However confirmation that cortisol levels increase after a heart attack could in future help doctors and patients in confirming the reliability of hair cortisol for diagnosis of stress.

What's involved?
On your regular clinic visit, a small (invisible and painless) hair sample will be taken from the crown of your head. Hair samples consisting of approximately 150 strands of hair, will be collected from the posterior vertex by the research nurse. The hair will be cut with scissors as close to the scalp as possible. The hair is then placed on an aluminium foil, with the scalp end clearly marked and the sample will be stored in a paper clips. The hair samples will be collected by the research nurse on the participant's visit to their respective clinics. At the end of the laboratory study, the data will be analysed. It may be necessary to repeat some of the experiments; thus hair samples will be stored until the results are published.

Will my taking part be kept confidential? Yes, only the nurses and doctors conducting the study will know your personal information which will be kept in a password protected computer. The hair samples will be given a unique lab number instead of the participant's name (unidentifiable) and stored safely at the Hair and Skin Research Lab. Paper-based records will be kept in a secure location and only accessible to personnel involved in the study, they will be kept for the duration of the study (2 years). Your identity will not be revealed without your permission. Your hair samples will be stored securely and only be used for this study; no other tests will be conducted without your permission and the approval by University of Cape Town's Research Committee.

We wish to invite you to be part of this study. If you have any queries or questions, please do not hesitate to contact the numbers listed below.

Yours Sincerely,
Professor Nonhlanhla P Khumalo,
Division of Dermatology, New Groote Schuur Hospital,
Observatory 7925, Cape Town

The use of hair cortisol as a biomarker of psychosocial stress in patients with recent acute myocardial infarction

I (full name)……
understand the project and hereby give consent to the investigators to use my hair samples for the purpose of furthering knowledge in the use of hair cortisol as a biomarker of stress. I understand that all my details will remain completely confidential.

I have read all of the above and anything I have not understood has been fully explained to me. I also understand that I may withdraw my participation at any stage from the study without repercussion.

Volunteer's signature:……Date:……

Place:……..

Witness (full name):……

Witness's signature:……

This study has been reviewed by the University of Cape Town's Research Committee and the research committee can be contacted on the following contact details:

Human Research Ethics Committee
Faculty of Health Science Human Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory, 7925
Email: sumayah.ariefdien@uct.ac.za

If I have questions, I may contact Sr Anthea Ndyenga at Tel: 021 4045269 or 4043376 Cell: 0761036437
OR – UCT's Faculty of Health Sciences, Human Research Ethics Committee at: 0214066338
OR – UCT's Faculty of Health Sciences, Human Research Ethics Committee at: 0214066338