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Cortisol and Testosterone in Hair as Biological Markers of Systolic Heart Failure

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pharmacology and Toxicology
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CORTISOL AND TESTOSTERONE IN HAIR AS BIOLOGICAL MARKERS OF SYSTOLIC HEART FAILURE.

(Thesis format: Integrated-article)

by

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of the requirements for the degree of
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Abstract

Congestive heart failure (CHF) is associated with increased stress and alterations in metabolism, favoring catabolism over anabolism. Hormonal profiles of patients with heart failure have been assessed using serum and saliva as matrices, which are only point measurements and do not provide long-term information. Scalp hair is a novel matrix that allows for measurement of hormones over a period of several months. We aimed to evaluate whether levels of cortisol and testosterone and their ratio (C/T) in hair correlate with severity of heart failure. We conducted a prospective study in ambulatory male patients with a left ventricular ejection fraction (LVEF) ≤40%. Hormone levels were measured using immunoassays in the proximal 2 cm of hair (representing approximately two months of systemic hormone exposure). Primary endpoints included the correlation of hair cortisol, testosterone, and C/T levels with the New York Heart Association (NYHA) class, LVEF, exercise capacity and NT-proBNP. The 44 CHF patients had a median hair level (range) of cortisol of 207 (117.7-1277.3) ng/g. Hair cortisol levels correlated positively with NYHA class (r=0.48, p=0.001) and negatively with treadmill stress test performance, (r=-0.37, p<0.05). The hair testosterone was 5.17 (2.39-24.64) ng/g and the C/T ratio was 39.89 (12.98-173.73). No associations were found between hair testosterone and C/T ratio and heart failure severity; however, the C/T ratio was higher in patients who required a CHF-related hospitalization than in patients who did not require this in the year following the inclusion in the study. Hair cortisol levels correlate with heart failure severity as assessed by the NYHA class and exercise capacity, while hair testosterone and C/T levels do not correlate with heart failure severity.

Keywords: Cortisol, Hair analysis, Heart failure, Prognosis, Testosterone

Co-Authorship Statement

Chapter 4 was written in collaboration with Dr. Stan Van Uum, Dr. Gideon Koren, and Dr. David Pereg. They were involved with the writing and editing of the manuscript prior to submission to the journal. As co-author, Dr. Pereg was involved in the patient recruitment for this study.

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TABLE OF CONTENTS

List of Figures and Tables

List of Abbreviations

3βHSD2 – 3β-hydroxysteroid dehydrogenase type 2

11β-HSD1 – 11β hydroxysteroid dehydrogenase type 1

11β-HSD2 – 11β hydroxysteroid dehydrogenase type 2

17βHSD1 – 17β-hydroxysteroid dehydrogenase 1

17βHSD3 – 17β-hydroxysteroid dehydrogenase 3

ACE – angiotensin converting enzyme

ACTH – adrenocorticotropic hormone

AMI – acute myocardial infarction

BMI – body mass index

C/T – cortisol over testosterone

CHF – congestive heart failure

CO – cardiac output

CRH – corticotropin releasing hormone

CVD – cardiovascular disease

DHEA – dehydroepiandrosterone

DHT - 5α-dihydrotestosterone

ELISA – enzyme linked immunosorbent assay

EPHESUS – Eplerenone Post-AMI Heart Failure Efficacy and Survival study

FSH – follicle stimulating hormone

GnRH – gonadotropin-releasing hormone

HF – heart failure

HR – heart rate

HPA – hypothalamic-pituitary-adrenal

LDL – low density lipoproteins

LH – luteinizing hormone

LVEF – Left Ventricular Ejection Fraction

NT-proBNP – N-terminal pro-Brain Natriuretic Peptide

NYHA – New York Heart Association

PSS – perceived stress scale

RALES – Randomized Aldactone Evaluation Study

stAR – Steroidogenic Acute Regulatory Protein

SV – stroke volume

WHR – waist-to-hip ratio

CHAPTER 1: INTRODUCTION

1.1 Heart Failure

Heart failure is a progressive disease that occurs when the heart is unable to eject sufficient blood to meet the metabolic demands of the body. There are approximately 500,000 Canadians living with heart failure and 50,000 new patients are diagnosed each year (Ross et al. 2006). There are two main types of heart failure, systolic and diastolic heart failure. This thesis will focus on systolic heart failure (also referred to congestive heart failure in this thesis). Systolic heart failure (HF) is commonly caused by uncontrolled hypertension, myocardial infarction, and/or valve dysfunction, consequently leading to decreased cardiac output (CO). CO is the product of stroke volume (SV) and heart rate (HR). The body reacts to decreased CO by releasing renin into the circulation from the juxtaglomerular cells of the kidney and stimulating the sympathetic nervous system to increase HR. Renin converts angiotensinogen to angiotensinogen I, which is subsequently converted to angiotensinogen II by angiotensin converting enzyme (ACE). Angiotensin II causes arterial and venous constriction increasing blood pressure. Angiotensin II and chronic high blood pressure causes cardiac remodelling where the cardiac myocytes hypertrophy, leading to decreased contractility of the heart and SV as well. Due to the decreased SV, the CO decreases further, more renin is released and a vicious cycle ensues. HF patients often have resting tachycardia in an attempt to compensate for decreased SV. Angiotensin II also stimulates the secretion of aldosterone from the adrenal cortex and endothelin from endothelial cells. Endothelin further contributes to the high blood pressure by constricting blood vessels. Aldosterone causes the distal tubules and collecting duct of the kidneys to reabsorb sodium and water, exacerbating the hypertension. Due to the chronic

retention of water and salt, edema occurs including in the lungs of systolic heart failure patients, which increases the strain on an already weak heart. Additionally, aldosterone and endothelin have been shown to contribute to the cardiac remodelling described above (Agapitov et al. 2002, Barr et al. 1995, Farquharson et al. 2000). The effect of cortisol and testosterone in heart failure are discussed in Chapter 2.
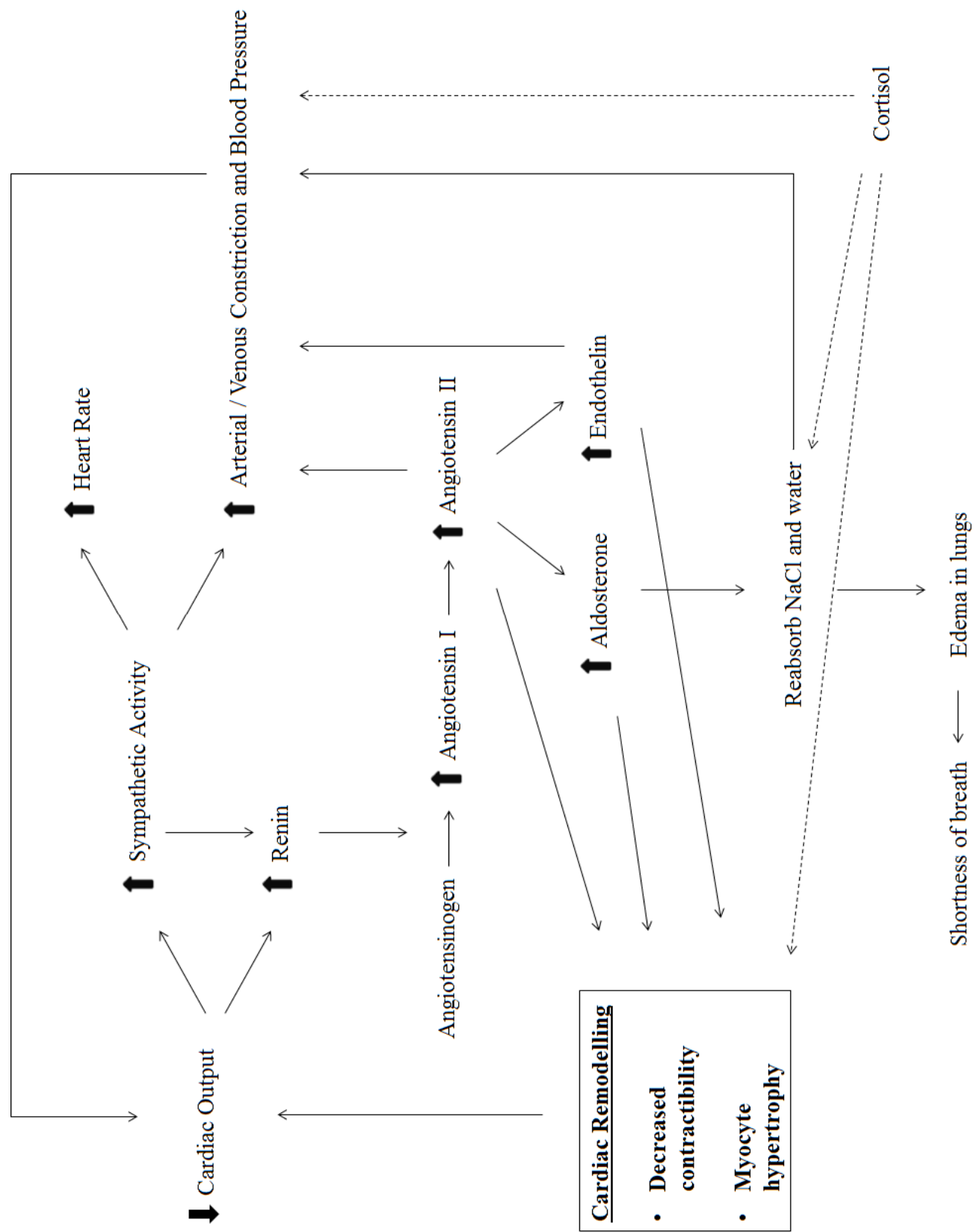
Figure 1: Pathophysiology of Systolic Congestive Heart Failure.

1.2 Prognostic markers of heart failure

Accurate classification of HF patients with respect to prognosis would potentially benefit from intensive medical therapy and/or cardiac transplantation is a major challenge. Currently, there are several clinical and laboratory predictors of survival including the New York Heart Association (NYHA) functional class, left ventricular ejection fraction (LVEF), exercise capacity, and N-terminal pro-Brain Natriuretic Peptide (NT-proBNP) levels.

1.2.1 New York Heart Association Class

There are four classes in the NYHA criteria. Class I HF is associated with no limitation and symptoms while performing ordinary activities; however during exercise, limitations and symptoms become apparent. Class II HF is characterized by slight limitation on ordinary activity resulting in fatigue and palpitations. Class III HF patients have no symptoms at rest, but fatigue easily with less than normal daily activity. Class IV HF is associated with symptoms at rest and the inability to carry out any physical activity. A limitation of the NYHA score is that it is not very reproducible and does not reliably predict the exercise capacity (Raphael et al. 2007).

1.2.2 Left Ventricle Ejection Fraction

LVEF is a measurement of the percentage of blood leaving the heart each time it contracts. LVEF can be measured by echocardiography or radionuclide angiography. Radionuclide angiography involves injection of a radioisotope into the blood to detect its flow through the left ventricle. Healthy individuals typically have ejection fractions between 50% and 65% (Kumar et al. 2009). In HF patients, LVEF falls below 35 to 40 percent. Interestingly, there

is no predictable relationship between symptoms or exercise capacity and LVEF; however in general, lower LVEFs are associated with worse prognosis (Wong et al. 2004).

1.2.3 Exercise capacity

Exercise capacity is reduced in patients with HF and has been shown to outperform traditional markers of heart failure prognosis (Myers et al. 2002, O'Connor et al. 2012). Treadmill stress test protocols such as the Bruce protocol and Naughton protocol can be used to assess exercise capacity. The Bruce protocol is carried out by walking on a treadmill. The initial speed of the treadmill is set to 2.7 km/h and the incline is set to 10%. After every 3 minutes, the end of one stage, both the speed and inclination of the treadmill is increased. The test is generally stopped when ECG machine shows abnormal changes or when a patient reaches his peak heart rate. Patients' heart rates and perceived exertion are taken every minute. Blood pressure is taken at the end of each stage. The Naughton protocol is less intense compared to the Bruce protocol. It begins with a two minute warm up with the treadmill set to 1.6 km/h and the incline is set to 0%. The treadmill is subsequently set to 3.2 km/h and does not change for the remainder of the test. The incline increases by 3.5% every two minutes.

1.2.4 N-terminal pro-Brain Natriuretic Peptide

NT-proBNP is measured in plasma and is typically higher in patients with worse outcome. The addition of NT-proBNP measurement in an emergency department can improve the diagnostic accuracy of CHF compared to standard clinical judgment alone in patients presenting with dyspnea. In the COPERNICUS study, all-cause mortality and hospitalization were significantly higher in patients with higher plasma NT-proBNP levels than the median (Hartmann et al. 2004). The Australia/New Zealand Heart Failure trial demonstrated similar

findings (Richards et al. 2001). There is evidence to suggest NT-proBNP may be useful for titrating therapy of HF in the absence of worsening clinical symptoms. A trial randomly assigned 278 patients to multidisciplinary care, NT-proBNP guided care, or usual care (Berger et al. 2010). The NT-proBNP guided group had significantly reduced hospitalization days compared to the other two groups. Mortality at one year was significantly lower in the NT-proBNP guided care and multidisciplinary care compared to the usual care group. Another study with 364 patients found similar results for patients age 60 to 75, but not over 75 years of age (Lainchbury et al. 2009).

1.3 Cortisol

Cortisol is a glucocorticoid produced by the zona fasciculata of the adrenal gland and is released in response to mental and physical stress. It is synthesized from *de novo* and circulating cholesterol. The first step in steroidogenesis involves the transport and loading of cholesterol into the mitochondria by the Steroidogenic Acute Regulartory Protein (stAR) followed by conversion to pregnenolone by the P450scc enzyme (Miller and Auchus 2011). Once pregnenolone is produced from cholesterol, it may be converted to progesterone or 17α-hydroxypregnenolone. For the biosynthesis of cortisol, both progesterone and 17α-hydroxypregnenolone is converted to 17α-hydroxyprogesterone by P450c17 and 3β-hydroxysteroid dehydrogenase 2 (3βHSD2), respectively.   17α-hydroxyprogesterone is subsequently converted to 11-deoxycortisol by P450c21, which is converted to cortisol by P450c11β (Figure 2).

The release of cortisol is regulated by the hypothalamic-pituitary-adrenal (HPA) axis and acts to increase blood glucose and blood pressure, decrease inflammation and bone formation, and aid in fat metabolism. In response to stress, the paraventricular cells of the hypothalamus secretes corticotropin-releasing hormone into the hypophyseal portal system, triggering cells of the anterior pituitary gland to secrete adrenocorticotropic hormone into the bloodstream, which in turn triggers the adrenal cortex to release cortisol into the blood. Cortisol can down-regulate its own release through negative feedback on the pituitary and hypothalamus (Figure 3). It travels through the blood as both free cortisol (active) and bound to cortisol-binding globulin (inactive) (90%) (Dunn et al. 1981). Since free cortisol is lipophilic, it diffuses through the cell membrane and binds to its glucocorticoid receptor in the cytoplasm. This ligand-receptor complex subsequently enters the nucleus and regulates the transcription of target genes by binding to hormone response elements. In addition to the stress response, cortisol follows a diurnal profile – levels of cortisol peak in the morning (8 AM) and reach its lowest level at night (12 PM). The reason for this diurnal rhythm is not well understood.

Cortisol is metabolized by the 11β-hydroxysteroid dehydrogenase system. Type 1 11β-hydroxysteroid dehydrogenase (11β-HSD1) reduces biologically inert cortisone to biologically active cortisol, while type 2 11β-hydroxysteroid dehydrogenase (11β-HSD2) oxidizes cortisol to cortisone. Cortisol in high concentrations can cross-react and activate the mineralocorticoid receptor consequently leading to aldosterone-like effects such as hypokalemia, hypernatremia, and hypertension (Brunner et al. 2002). Since cortisone is less active than cortisol, the 11β-hydroxysteroid dehydrogenase system is crucial to prevent overstimulation of the mineralocorticoid receptor. Clearance of cortisol involves the conversion of cortisol to dihydrocortisol in the liver. Dihydrocortisol is further metabolized to tetrahydrocortisol, which is

subsequently conjugated with glucoronic acid to form tetrahydrocortisol glucuronide before excretion in urine.
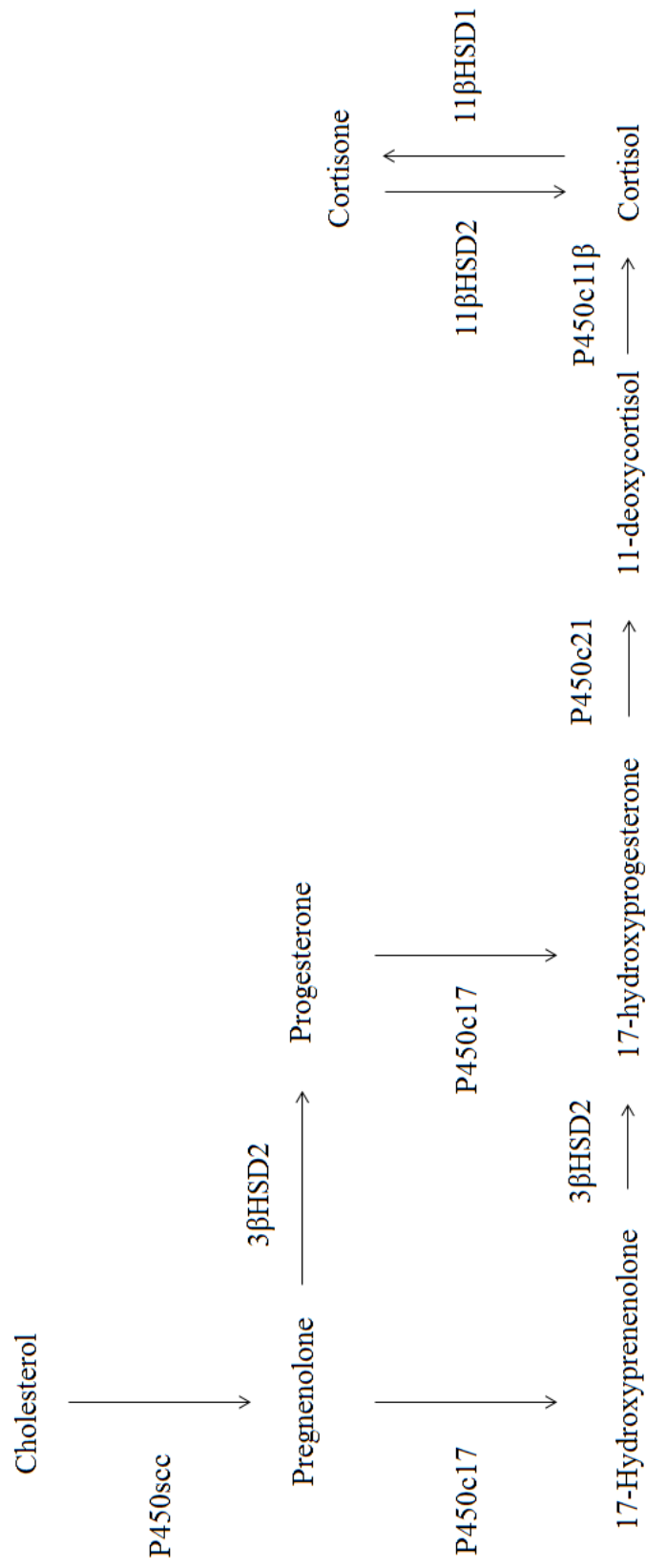
Figure 2: Biosynthesis of cortisol. 3βHSD2, 3β-hydroxysteroid dehydrogenase (Adapted from Miller and Auchus 2011)
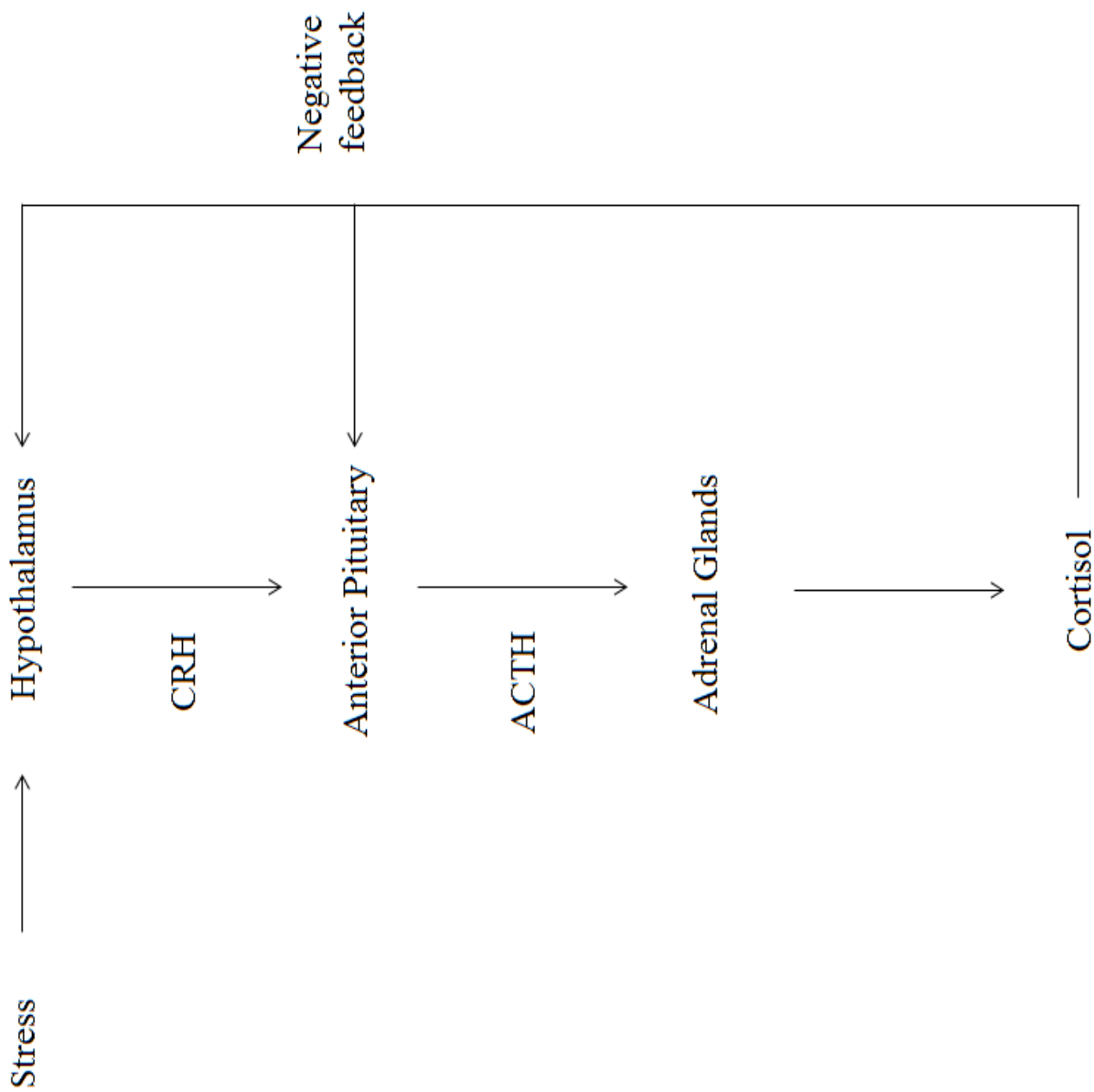
Figure 3: Hypothalamic-pituitary axis. CRH, corticotrophin releasing hormone; ACTH, Adrenocorticotrophic hormone

1.4 Testosterone

Testosterone is an androgen primarily synthesized by Leydig cells in the testes of males and thecal cells in the ovaries of females. The biosynthesis of testosterone is similar to the biosynthesis of cortisol; however 17α-hydroxypregnenolone is converted to dehydroepiandrosterone (DHEA) by P450c17 (Miller and Auchus 2011). In Leydig cells, DHEA is converted to androstenedione by 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2) and then to testosterone by 17β-hydroxysteroid dehydrogenase 3 (17βHSD3). DHEA can also be converted to androstenediol by 17βHSD3 and then to testosterone by 3βHSD2 (Figure 4). In thecal cells, androstenedione is converted to testosterone by AKR1C3 (minor pathway). The major pathway in thecal cells involves conversion of androstenedione to estrone by P450aro and subsequently estradiol by 17β-hydroxysteroid dehydrogenase 1 (17βHSD1) (Figure 5). Testosterone is cleared through the conversion of testosterone to androstenedione. Androstenedione is metabolized to androsterone and etiocholanolone, which are subsequently conjugated as sulfates and glucuronides for excretion in urine. Only a small portion of testosterone produced in the body is metabolized to testosterone glucuronide and is excreted as such in urine.

The release of testosterone is regulated by the HPA axis and acts to increase bone density and muscle mass and in males, develop sperm. In response to low testosterone, the hypothalamus secretes gonadotropin-releasing hormone (GnRH) triggering the pituitary gland to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH) into the bloodstream. LH triggers the interstitial cells of the testes to synthesize testosterone, whereas FSH stimulates spermatogenesis in Sertoli cells of testes. The Sertoli cells secrete inhibin to down-regulate the release of FSH and

LH. Testosterone also down-regulates its own release through negative feedback on the pituitary and hypothalamus (Figure 5). It travels through the blood as both free testosterone and sex-hormone binding globulin bound (25-50%). Like cortisol, free testosterone is lipophilic and diffuses through the cell membrane to bind to the androgen receptor in the cytoplasm. Free testosterone can also be reduced to 5α-dihydrotestosterone (DHT) in the cytoplasm by 5-alpha reductase. The ligand-receptor complex subsequently enters the nucleus and regulates the transcription of target genes by binding to hormone response elements.

Cholesterol

P450scc

↓

Pregnenolone

P450c17

↓

17-Hydroxyprenenolone

P450c17

↓

DHEA   3βHSD2 ⟶ Androstenedione

17βHSD3 ↓   17βHSD3 ↓

3βHSD2

Androstenediol ⟶ Testosterone
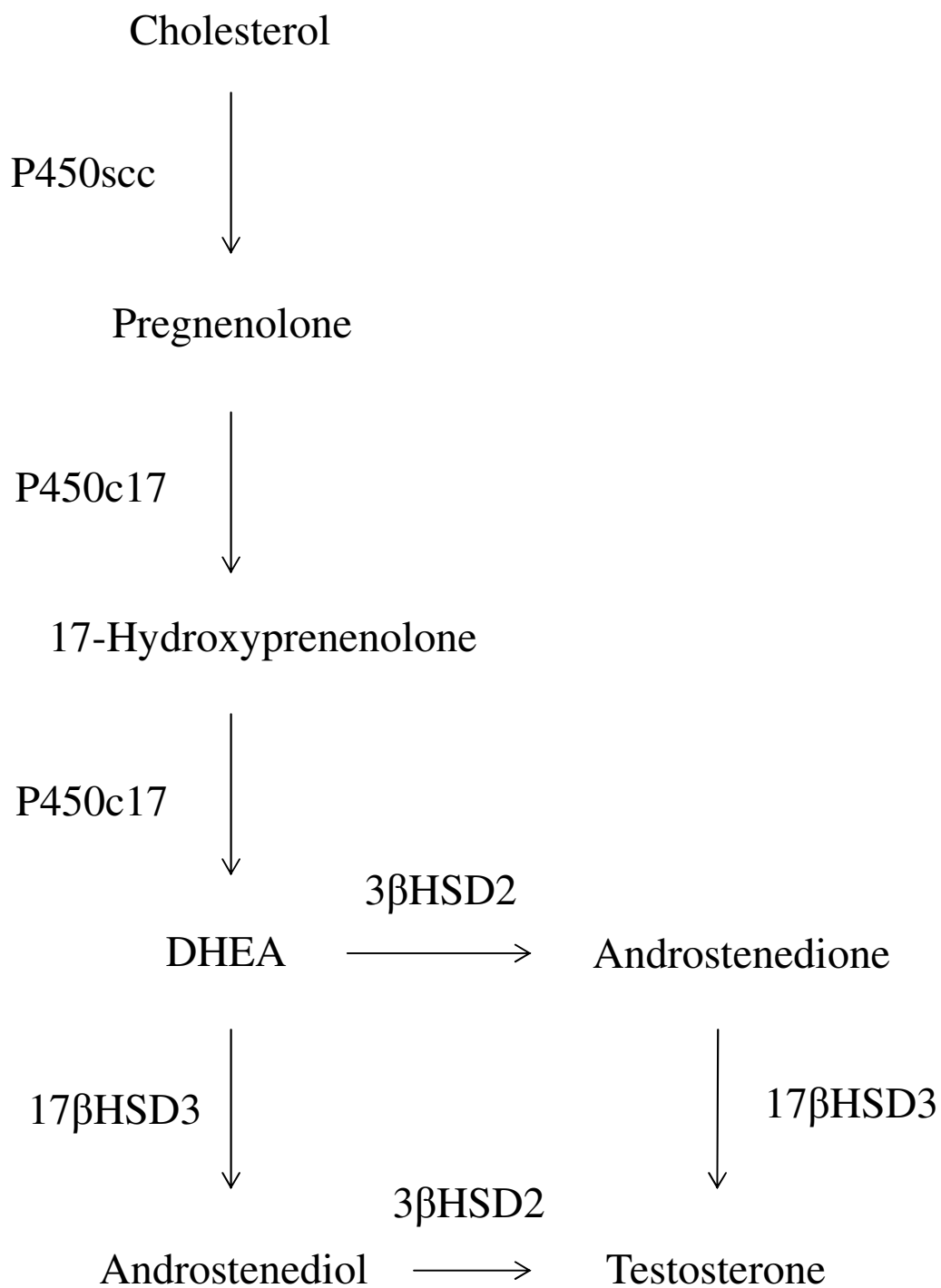
Figure 4: Biosynthesis of testosterone in Leydig cells. 17βHSD3, 17β-hydroxysteroid dehydrogenase 3; DHEA, Dehydroepiandrosterone; 3βHSD2, 3β-hydroxysteroid dehydrogenase 2 (Adapted from Miller and Auchus 2011)

Cholesterol

P450scc

Pregnenolone

P450c17

17-Hydroxyprenenolone

P450c17

DHEA → (3βHSD2) → Androstenedione → (P450aro) → Estrone

ARK1C3
(minor
pathway) ↓ 17βHSD1 ↓

Testosterone   Estradiol
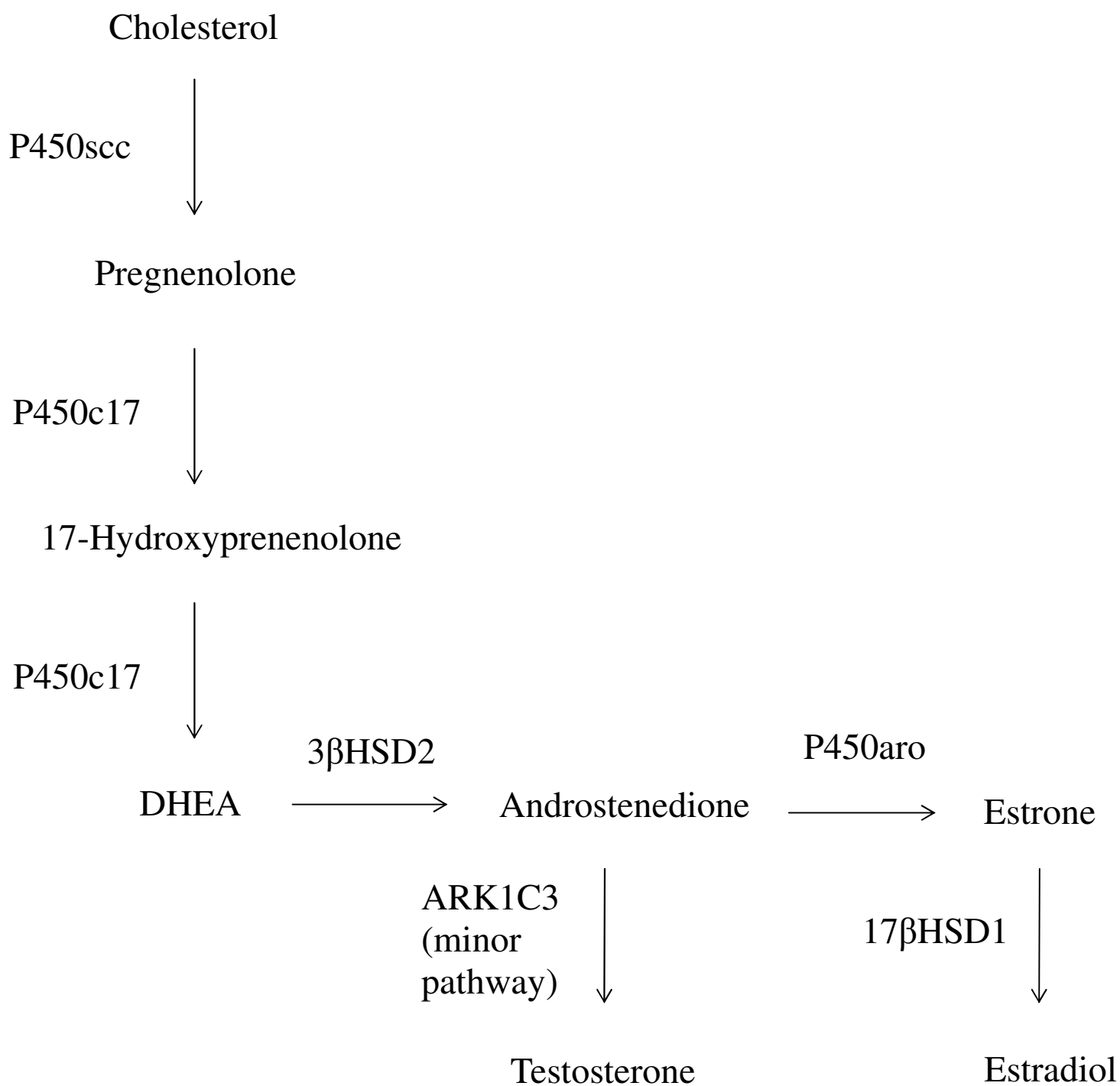
Figure 5: Biosynthesis of testosterone in thecal cells. 17βHSD1, 17β-hydroxysteroid dehydrogenase 1; DHEA, Dehydroepiandrosterone; 3βHSD2, 3β-hydroxysteroid dehydrogenase 2 (Adapted from Miller and Auchus 2011)
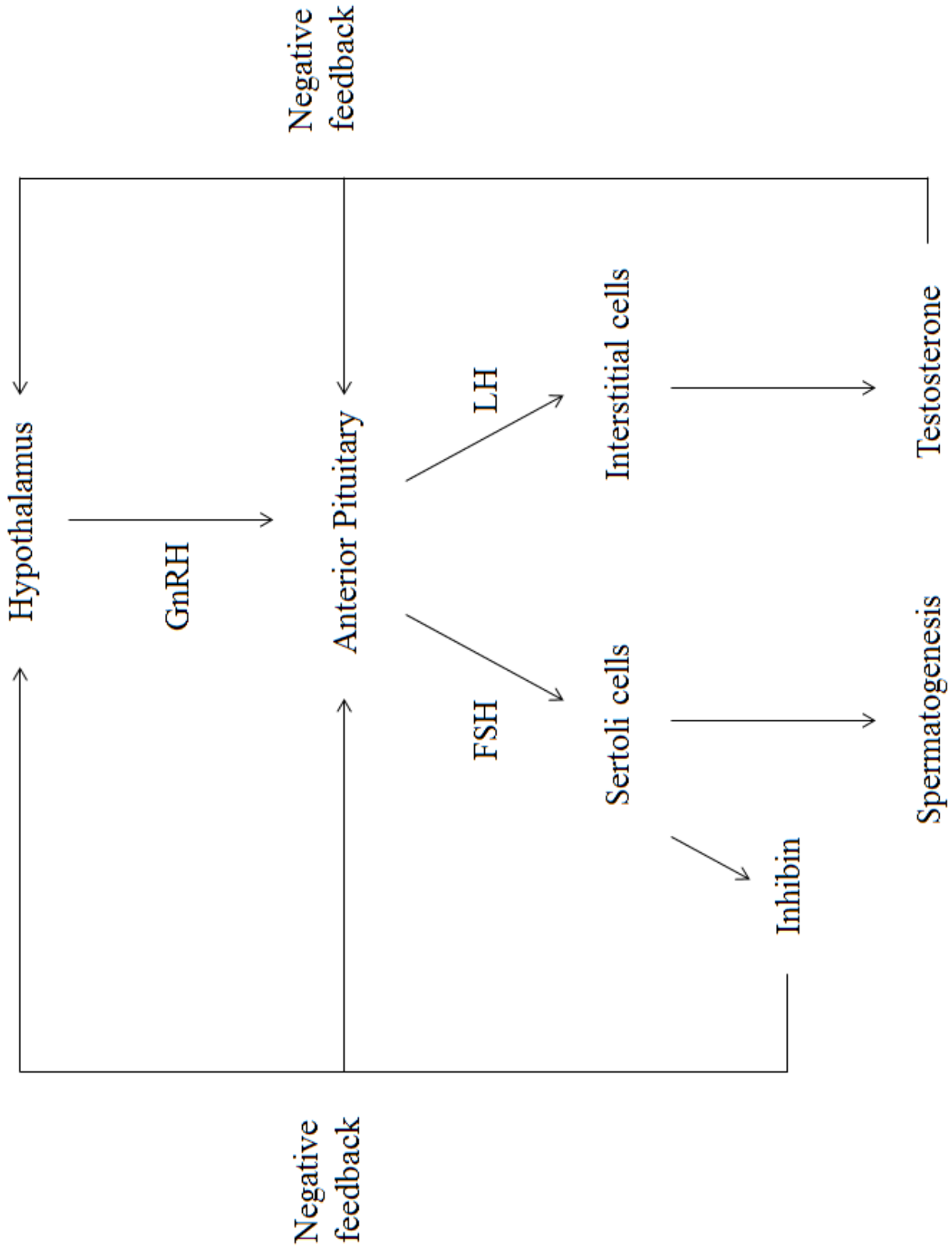
Figure 6: Hormonal control of testosterone. GnRH, gonadotropin releasing hormone; FSH, follicle stimulating hormone; LH, Luteinizing hormone

1.5 Hair Physiology

Hair fibers consist of several layers made up of the cuticle, cortex, and medulla (Figure 7). The cuticle is the outermost layer of the hair shaft and acts as a protective barrier for the cortex and medulla. The cortex is located between the medulla and cuticle and contains melanin, giving the hair its colour. The innermost layer is the medulla. It is hypothesized that drugs and hormones enter the hair via the medulla through passive diffusion from the blood (Boumba et al. 2006).

Hair grows out from hair follicles. The hair growth cycle is composed of three stages: Anagen (growth phase), catagen (transition phase), and telogen (resting phase). Anagen is the active growth phase of the hair. During this phase, cells at the root of the hair rapidly divide, subsequently elongating the hair strand. The anagen phase usually lasts around two to six years for human scalp hair before transitioning to the catagen phase (Boumba et al. 2006). In the catagen phase, blood supply is cut off from cells that produce new hair, consequently ending cell division (Boumba et al. 2006). This phase approximately lasts for two to three weeks before transitioning into the telogen phase. In the telogen phase, hair growth has stopped completely and germ cells below the root will produce a new hair strand to force the old strand out. This phase lasts for approximately 3 months.

In general, individual hair strands are in different stages of the growth cycle. A healthy head consists of 80–90% of hair follicles in the anagen phase, 2% in the catagen phase and 10–18% in the telogen phase (Harkey 1993). Scalp hair grows at a rate of 0.6 to 1.4 cm per month (Wennig et al. 2000).
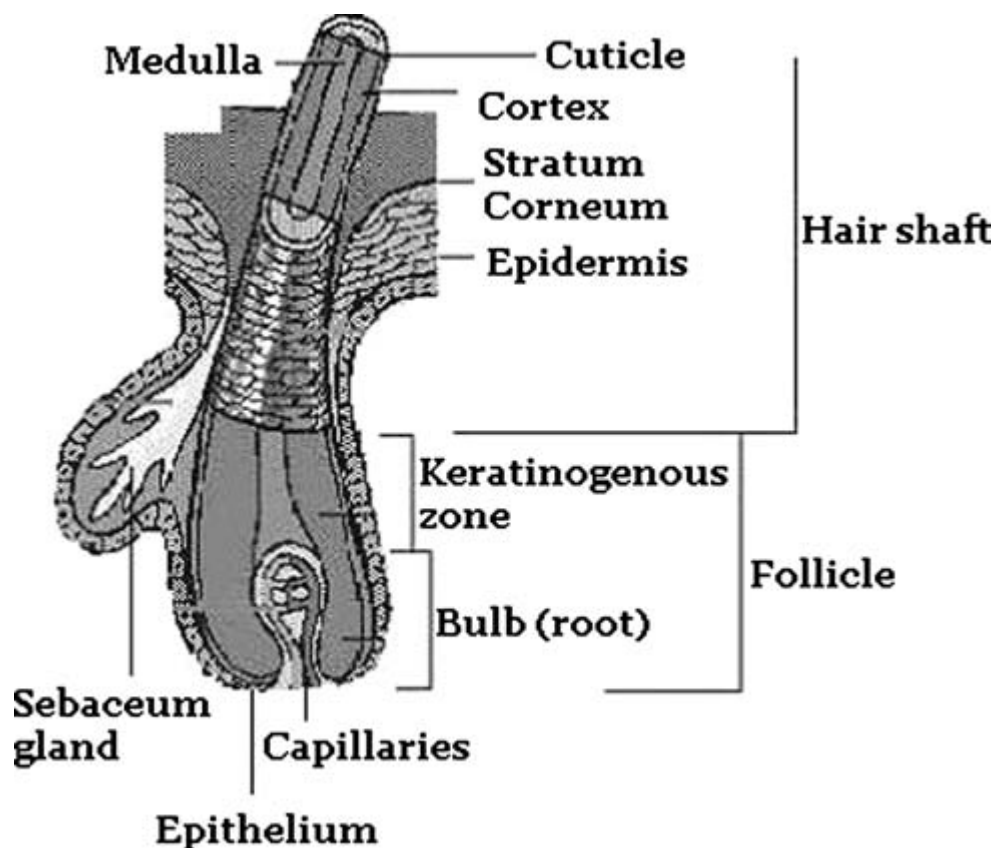
Figure 7: Hair structure (adapted from Boumba et al. 2006)

1.6 Incorporation of compounds into hair

Hair analysis has been demonstrated to accurately reflect exposure to drug abuse, environmental toxins and exogenous hormones (Raul et al., 2004; Villain et al., 2004). The incorporation of substances into hair still remains unclear; however, currently accepted models assume substances are incorporated into hair from passive diffusion from the blood, deep skin compartments, sweat, sebum, and the external environment (Figure 8). In general, lipophilic and basic molecules are more easily incorporated into the hair (Pragst et al. 2006). This is because

lipophilic substances can easily diffuse through membranes and the intracellular pH of melanocytes is more acidic than the pH of plasma.
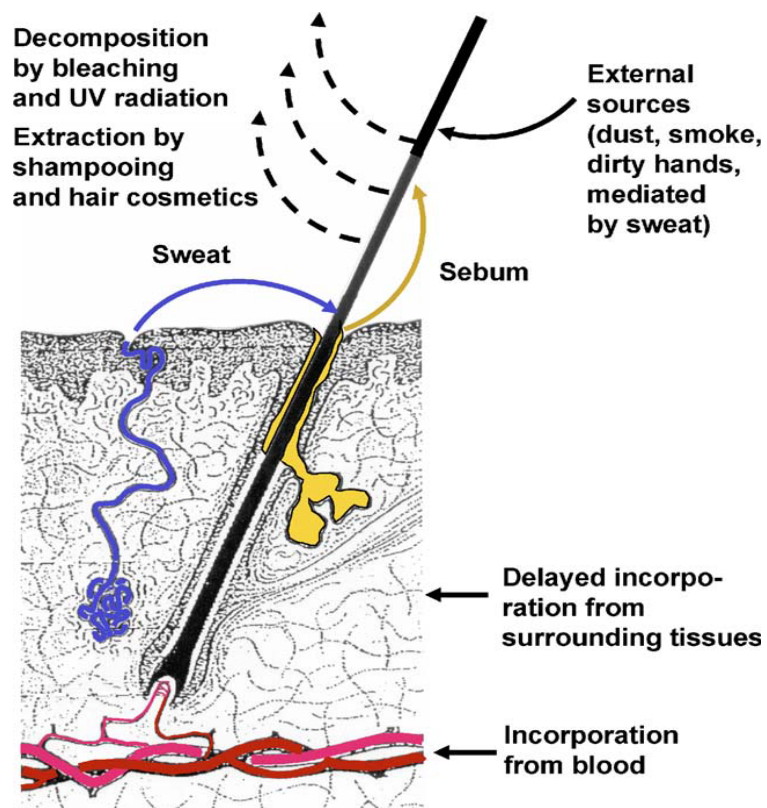
Figure 8: Mechanisms of incorporation of compounds into hair (adapted from Pragst et al. 2004)

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CHAPTER 2: LITERATURE REVIEW AND STUDY RATIONALE

2.1 Hair cortisol as a measure of stress

One of the first studies to suggest that hair cortisol could be used as a biological marker of stress was done in macaque monkeys (Davenport et al. 2006). In this initial work, monkeys were relocated to a new home and hair cortisol levels were assessed pre- and post- move. Hair cortisol concentrations were about 60% higher post-move compared to pre-move, reflecting the stressful nature of moving to a new home. The hair cortisol levels returned to pre-move concentrations one year following the relocation.

Over the past few years, we and other groups have conducted studies investigating the use of hair cortisol as a biological marker of chronic stress in humans. In 2007, our laboratory showed that infants who were hospitalized after birth had significantly higher hair cortisol concentrations compared to healthy term infants (Yamada et al. 2007). Infants in the neonatal intensive care unit showed that for each extra day on the ventilator, hair cortisol levels increased on average of 0.2 nmol/g. These results suggest that hair cortisol could potentially be a valid biological marker to assess chronic neonatal stress.

In another study, our group showed that hair cortisol concentrations in pregnant women correlated with perceived stress as measured by the Perceived Stress Scale (PSS) (Kalra et al. 2007). In brief, the PSS is a validated 10-item, psychological questionnaire to quantify perceived stress. In 2008, we demonstrated that hair cortisol levels were significantly elevated in patients with severe chronic pain compared to controls (Van Uum et al. 2008). Further, the PSS scale determined that subjective stress was greater in patients with chronic pain; however, correlation

between the PSS scale and hair cortisol levels in this study did not reach statistical significance (p = 0.08).

A comparison between unemployed and employed individuals found that unemployed individuals had significantly higher hair cortisol concentrations (Dettenborn et al. 2010). These individuals also had increased levels of perceived stress; however, subjective measures of perceived stress did not correlate with hair cortisol. This study also discusses that the lack of association between cortisol levels and subjective measures of stress is not uncommon. There is evidence that suggests that associations between cortisol responses and self-reported stressors are mixed (Al'Absi et al. 1997; Buchananet al. 1999; Cohen et al. 2000; Oswald et al. 2004).

In 2010, Stalder et al. compared hair cortisol levels in abstinent alcoholics, alcoholics in acute withdrawal, and controls (Stalder et al. 2010). This group found that hair in alcoholics in acute withdrawal had four times the levels cortisol than those of abstinent alcoholics and controls. There were no differences in hair cortisol concentrations between the latter two groups.

Another study investigating hair cortisol in young adults found that mean cortisol levels in 99 university students were significantly related to serious life events after multivariate analysis (Karlen et al. 2011). Of note, out of the four outliers with extremely high hair cortisol levels, this group managed to contact two students who reported serious psychological problems (anorexia and severe anxiety disorder). Investigators also demonstrated a very weak negative correlation between hair cortisol and perceived stress (r = -0.061, p < 0.05).

Shift workers at a young age (less than 40 years) have also been shown to have higher levels of hair cortisol compared to day workers (Manenschijn et al. 2011). However, at an older age (greater than 40 years) there was no difference between shift and day workers. In this study,

hair cortisol levels were positively related to BMI, a finding in line with a recent study conducted by our group (Chan et al. *submitted*). Manenschijn et al. suggest that the elevated cortisol concentrations and elevated BMI may contribute to the prevalence of metabolic syndrome and increased cardiovascular risk found in shift workers.

Elevated hair cortisol levels has also been shown in endurance athletes (n = 304) compared to controls (n = 70) (Skoluda et al. 2012). Interestingly, a dose-response association was found between training volume and hair cortisol levels suggesting that repeated physical stress of intensive training and competitive races among endurance athletes is associated with elevated cortisol exposure over prolonged periods of time.

2.2 Cortisol, Stress, and Cardiovascular Risk

Numerous studies have been conducted associating salivary, urinary and serum cortisol with cardiovascular disease (CVD) risk and prognosis. Recent salivary cortisol studies have demonstrated an association between heightened cortisol reactivity to stress and incident hypertension and coronary artery calcification, suggesting increased HPA axis activation may influence the risk of CVD (Hamer et al. 2012a; Hamer et al. 2012b). The prognostic value of serum cortisol levels has also been evaluated in a large study of patients with chronic heart failure who were admitted to hospital due to various causes (Guder et al. 2007). This study demonstrated that higher serum levels of cortisol were independent predictors of increased mortality risk. Another recent study found that elevated plasma cortisol is associated with a greater prevalence of ischemic heart disease, independent of conventional risk factors (Reynolds

et al. 2010). Further, high urinary cortisol secretion strongly predicts cardiovascular death among persons both with and without pre-existing cardiovascular diseases (Vogelzangs et al. 2010).

There is increasing evidence suggesting that cortisol may be involved directly in the pathological processes that lead to heart failure progression by activating the cardiac mineralocorticoid receptors similar to aldosterone (Arriza et al. 1987; Young et al. 2003). In both the Randomized Aldactone Evaluation Study (RALES) and the Eplerenone Post-AMI Heart Failure Efficacy and Survival study (EPHESUS), the beneficial effects of aldosterone blockade were observed despite normal plasma levels of aldosterone (Pitt et al. 1999; Pitt et al. 2001). This finding supports the concept that other ligands, such as cortisol, may activate the cardiac mineralocorticoid receptor in pathological conditions. Cortisol and aldosterone exhibit a similar affinity to the mineralocorticoid receptor, and free cortisol circulates at systemic concentrations 2 orders of magnitude higher than aldosterone. This theory may suggest that patients with high cortisol levels may benefit more from mineralocorticoid receptor blockade. A recent study found in patients who were not treated with eplerenone, the total concentration of cortisol metabolites were associated with cardiac remodelling, while such a relation was not found in patients treated with eplerenone (Weir et al. 2011).

2.3 Testosterone and Cardiovascular Risk

Similar to cortisol, testosterone studies have primarily used plasma as a matrix of measuring hormone levels. A study of 208 men with HF demonstrated that men with CHF have significantly reduced levels of testosterone (Jankowska et al. 2006). Furthermore, these deficiencies in testosterone have been associated with increased mortality. This study also

showed a significant stepwise decrease in the levels of both total testosterone and estimated free testosterone with worsening severity of CHF. However, it is not known whether these deficiencies contributed to the severity of HF, were themselves the result of advanced HF, or whether they were markers of the severity of general chronic illness. A recent study found that low testosterone predicted mortality from CVD, but not from other causes (Hyde et al. 2012).. Another group conducted a randomized, double-blind, placebo-controlled parallel trial of testosterone replacement therapy at physiological doses in 76 men with heart failure over 12 months (Malkin et al. 2010). Exercise capacity and symptoms significantly improved at least one functional class with testosterone therapy compared with placebo, suggesting testosterone replacement therapy improves functional capacity and symptoms in men with moderately severe heart failure. A low testosterone level is associated with increased carotid intima-media thickness in men with low-grade inflammation (Soisson et al. 2012) and impaired endothelial function (Empen et al., 2012). Moreover, free testosterone was shown to be negatively correlated with Framingham risk score (a measure to estimate 10-year CVD risk), age, and BMI, but positively correlated with total cholesterol, LDL, and current smoking status (Chock et al. 2012). These associations suggest that low testosterone may suggest increased risk of CVD. Although an analysis of the Framingham population did not find an association between low testosterone and cardiovascular risk (Haring et al. 2013), a low serum testosterone was associated with increased risk of all-cause mortality (Haring et al. 2010).

2.4 Cortisol/Testosterone Ratio and Cardiovascular Risk

In light of the association between high cortisol and increased cardiovascular risk factors and all-cause mortality, coupled with the association between low testosterone and all-cause mortality and possibly cardiovascular risk, the ratio of cortisol over testosterone might be particularly interesting. The clinical significance of the cortisol over testosterone ratio in serum has been demonstrated in the Caerphilly study, showing a strong positive correlation between this ratio and both the insulin resistance syndrome and incident ischemic heart disease (Smith et al. 2005). Additionally, the ratio of catabolic to anabolic steroid hormones, as measured by serum cortisol over dehydroepiandrosterone (DHEA) ratio, was higher in patients with CHF compared to controls (Anker et al. 1997). This metabolic shift toward catabolism in congestive heart failure (CHF) patients may cause progressive exercise intolerance and cardiac cachexia (Anker et al. 1997). Based on these data it is possible that the hair cortisol-testosterone ratio may be useful in population studies investigating the risk of metabolic syndrome and cardiovascular events.

2.5 Knowledge Gap

To date, no studies have evaluated testosterone, measured in hair, and its association with CVD risk or prognosis. Only two studies have evaluated cortisol measured in hair to determine risk or prognosis of CVDs. In 2010, our laboratory conducted a study on hair cortisol and the risk for acute myocardial infarction (AMI) in adult men and demonstrated that after controlling for all other risk factors, hair cortisol content remained the strongest predictor (Pereg et al. 2011).

A recent study demonstrated that higher hair cortisol levels were associated with a history of cardiovascular disease (Manenschijn et al. 2013). This study found that participants in the highest hair cortisol quartile had a 2.7 times increased risk of CVD. Importantly, this risk was similar to the effect of traditional cardiovascular risk factors such as hypertension, obesity, and dyslipidemia, suggesting that long-term elevated cortisol may also be an important risk factor. There were no associations between hair cortisol levels and non-cardiovascular diseases. There have been no studies to date that have examined hair cortisol and testosterone specifically in heart failure patients and their association with clinical outcomes.

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CHAPTER 3: HYPOTHESIS AND OBJECTIVES

3.1 Primary Research Question

Does cortisol, testosterone, and their ratio (C/T), measured in hair, reflect heart failure status and prognosis?

3.2 Hypothesis

We hypothesized that high hair cortisol and low hair testosterone levels serve as biological markers of poor congestive heart failure prognosis.

3.3 Objective

To evaluate whether levels of hair cortisol and testosterone and the ratio of catabolic to anabolic steroid hormones, as measured by hair cortisol over testosterone (C/T) ratio, correlate with heart failure severity and prognosis in ambulatory patients with stable chronic systolic heart failure.

CHAPTER 4: HAIR CORTISOL AND TESTOSTERONE IN PATIENTS WITH SYSTOLIC HEART FAILURE

This section has been published:

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4.1 Introduction

Progression of chronic congestive heart failure (CHF) is associated with activation of neuro-endocrine stress response systems including the hypothalamic-pituitary-adrenal axis that modulates the production and secretion of glucocorticoids including cortisol from the adrenal cortex (Güder et al., 2007; Brotman et al., 2007). The prognostic value of serum cortisol levels has been evaluated in a single large study of patients with chronic heart failure who were admitted to hospital due to various causes (Güder et al., 2007). This study demonstrated that higher serum levels of cortisol were independent predictors of increased mortality risk. However, it is possible that the single serum cortisol measurement may have been influenced by the physical stress due to the acute illness and/or the emotional stress associated with the admission itself.

Furthermore, deficiency of anabolic sex steroids is common among patients with CHF, affecting up to two thirds of patients (Moriyama et al., 2000; Jankowska et al., 2006). Several studies have demonstrated that in patients with CHF a low serum testosterone is an independent predictor of mortality (Jankowska et al., 2006; Güder et al., 2010; Wehr et al., 2011). Furthermore, low serum testosterone has been correlated with other poor prognostic factors of heart failure including lower left ventricular ejection fraction (LVEF) (Jankowska et al., 2006), poor exercise capacity (Jankowska et al., 2009; Pastor-Pérez et al., 2011), high New York Heart Association (NYHA) class (Jankowska et al., 2006; Güder et al., 2010) and increased levels of NT-proBNP (Jankowska et al., 2006; Jankowska et al., 2009).

Additionally, the ratio of catabolic to anabolic steroid hormones, as measured by serum cortisol over dehydroepiandrosterone (DHEA) ratio, was higher in patients with CHF compared to control patients (Anker et al., 1997). Further, the Caerphilly study found a positive linear association between the serum C/T ratio and future incident ischemic heart disease (Smith et al., 2005). While these studies were well designed, they all share the same limitation – the assessment of hormone levels was based on a single measurement. Given the level of intra-individual variability in hormone levels, one serum sample may not be sufficient to characterize an individual's hormone levels (Smith et al., 2005; Brambilla et al., 2007; Brambilla et al., 2009). Cortisol and testosterone are typically measured in blood, urine, or saliva; however, these matrices only provide systemic hormone exposure over a period of 24 hours or less, are subject to diurnal variation (blood and saliva), can be invasive, and may require repeated measurements. The measurement of these hormones in scalp hair is a novel method allowing retrospective assessment of average cortisol and testosterone exposure over a period of several months using

only a single sample collection. Hair grows approximately 1 centimeter per month and therefore hair analysis reflects long-term endogenous production of cortisol and testosterone. For example, cortisol measurement from the most proximal 3 cm of hair represents the most recent 3 months of exposure (similar to the assessment of glucose levels using hemoglobin A1C). For the first time, this provides a reliable mode for measuring the accumulation of cortisol over time (Sauvé et al. 2007; Thomson et al., 2010). Several reports have demonstrated an association between high hair cortisol levels and various clinical conditions in both animal models and in humans (Yamada et al., 2007; Van Uum et al., 2008; Davenport et al., 2008; Stalder et al., 2010; Gow et al., 2011; Pereg et al., 2011; Manenschijn et al., 2012). A recent study assessed the relation between hair cortisol and presence of metabolic syndrome in 1258 employees of a large company undergoing a voluntary health assessment (Stalder et al. 2013). Participants whose hair cortisol levels fell into the third and fourth quartile had an odds ratio for having metabolic syndrome of 1.71 and 2.42, respectively, as compared to the first quartile. Another recent study in community dwelling elderly patients demonstrated that higher hair cortisol levels were associated with a history of cardiovascular disease (Manenschijn et al. 2013). This study found that participants in the highest hair cortisol quartile had a 2.7 times increased risk of CVD compared to the lowest quartile. Importantly, this risk was similar to the effect of traditional cardiovascular risk factors such as hypertension, obesity, and dyslipidemia, suggesting that long-term elevated cortisol may also be an important risk factor. However, this study cannot determine if increased hair cortisol also predicts future cardiovascular events. Furthermore, we have shown that testosterone levels in hair are lower in individuals with hypogonadism and normal in hypogonadal subjects on testosterone treatment (Thompson et al., 2009). The longitudinal assessment of cortisol and testosterone levels over time using the hair technique

may be more reliable than a single random serum sample for the assessment of chronic heart failure status and prognosis.

Our objective was to evaluate whether levels of hair cortisol and testosterone and the ratio of catabolic to anabolic steroid hormones, as measured by hair cortisol over testosterone (C/T) ratio, correlate with heart failure severity and prognosis in ambulatory patients with stable chronic systolic heart failure.

4.2 Methods

The study was approved by the research ethics committee at the Meir Medical Center and written informed consent was obtained from each participant. The study was registered in the ClinicalTrials system (ID: NCT01139697).

We included male patients above the age of 18 years with at least moderately reduced left ventricular systolic function defined as left ventricular ejection fraction (LVEF) ≤ 40%. All participants were routinely followed-up in either the outpatient cardiology clinic or cardiac rehabilitation center at the Meir Medical Center. Since we focused on patients with stable CHF, those with any hospital admission within the last 3 months prior to enrolment were excluded. Other exclusion criteria included any testosterone treatment, glucocorticosteroid treatment (either systemic or topical) within the last 6 months, diagnosis of Cushing's or Addison's disease, dyed hair, and morbid obesity (defined as body mass index >35 kg/m^2). Patients for whom a hair sample of at least 2 cm from vertex posterior could not be obtained were excluded for technical reasons.

All patients underwent a detailed standardized physical examination and clinical assessment including New York Heart Association (NYHA) class determination. Furthermore, hair samples and blood tests for basic chemistry, complete blood count, highly sensitive C-reactive protein and NT-proBNP were drawn at enrolment. A treadmill exercise test was performed on all patients that were capable of completing a meaningful test. Following enrollment, we determined one year all-cause mortality and CHF-related hospitalizations data by collecting National Social Security records and through telephone follow-up. Patients with CHF were admitted to the hospital if CHF was the first and main diagnosis in the hospital discharge summary and if any of the following criteria were present: Respiratory distress or pulmonary edema, hypoxia, significant edema, syncope, or hypotension.

End points

The primary endpoints included the correlation of cortisol, testosterone, and the C/T ratio in hair with the New York Heart Association (NYHA) class, left ventricular ejection fraction LVEF, exercise capacity as measured by a treadmill exercise test and serum levels of NT-proBNP. Secondary endpoints included all-cause mortality and CHF-related hospitalizations at 1 year.

Quantification of hair levels of cortisol and testosterone

Scalp hair samples were obtained by a standard protocol established by the Motherisk Laboratory (Sauvé et al. 2007; Yamada et al. 2007; Thomson et al., 2010). The two most proximal centimeters of scalp hair, thought to represent two months of systemic hormone exposure in healthy individuals, were measured, cut, and weighed to a minimum of 10 mg in a glass scintillation vial. Each hair section was washed with 3 mL of isopropanol, allowed to dry,

and subsequently minced with surgical scissors in 1 mL of methanol. Scintillation vials were then incubated for 16 hours at 50 ºC on a plate shaker set to 100 rpm. Next, the methanol was removed into a glass test tube and evaporated under a stream of nitrogen gas at 50 ºC. Following evaporation, the hair extract was reconstituted in 250 µL of phosphate-buffered saline and vortexed for 30 seconds. Hair cortisol and hair testosterone concentrations were determined using different Alpco Diagnostics (Salem, NH, USA) salivary enzyme immunoassay kits (ELISA) specific for each hormone. For hair cortisol quantification, 50 µL of each sample was pipetted onto the plate in duplicate. For hair testosterone quantification, 100 µL of each sample was pipetted onto the plate in duplicate as per the manufacturer's instruction.

Hair samples with known cortisol and testosterone levels were used as positive controls. Phosphate-buffered saline was used as a negative control to assess non-specific binding and the values obtained were subtracted from all other values prior to interpretation to ensure accuracy of measurement. The intra- and inter-day coefficients of variation for the cortisol immune assay were determined using a standard sample of hair measured over several weeks and was 5.4% (n=4) and 7.6% (n=4), respectively. The limit of detection of the cortisol immunoassay kit was 1.0 ng/ml (Alpco Diagnostics). Based on previous experience, cortisol levels above 1500 ng/g were excluded as they may possibly result from contamination or due to underlying Cushing (Thomson et al. 2010). For the testosterone immunoassay, the minimum required weight was 10 mg for each sample. The intra- and inter-day coefficients of variation was 7.8% (n=4) and 9% (n=4), respectively. The limit of detection of the testosterone immunoassay kit was 1.0 pg/ml (Alpco Diagnostics).

Other laboratory measurements

All other laboratory parameters, including basic chemistry (Boehringer Mannheim, Germany), and NT-proBNP (Roche diagnostics, Basel, Switzerland) were performed on fresh samples in the core laboratory facility of the Meir Medical Center.

Echocardiographic assessment

Complete Echo-Doppler study was performed on all participants within 1 month prior to enrollment. All studies were performed in our echocardiography laboratory according to a standardized protocol and were interpreted by the same echocardiographer.

Treadmill exercise testing

Stress test was symptom limited and patients were strongly advised not to use the handrails for support. All tests were conducted in the cardiac rehabilitation center at the Meir Medical Center. Either the modified Naughton or modified Bruce protocols (Working Group on Cardiac Rehabilitation & Exercise Physiology and Working Group on Heart Failure of the European Society of Cardiology, 2001) were used according to the attending cardiologist's assessment of the patient's functional capacity based on clinical evaluation and previous stress tests.

Statistical Analysis

IMB SPSS Statistics version 20 was used to analyze data. Mean and standard deviation (SD) were used to describe normally distributed variables. All cortisol, testosterone and C/T ratio data are presented as median and range. Spearman's rank correlation coefficient was used to assess the relationship between skewed continuous variables. The independent samples Kruskal-

Wallis test was used to compare hair cortisol concentrations among the NYHA classes. Differences in hair cortisol and testosterone concentrations between hospitalized and non-hospitalized patients were assessed using the Mann-Whitney U test. A univariate logistic regression was computed to obtain odds ratios for hair cortisol level and C/T ratio between patients who had a CHF-related hospitalization in the year following the inclusion in the study and those who did not. A p value < 0.05 was considered statistically significant.

4.3 Results

Participants

A total of 52 patients fulfilled all the inclusion criteria; however 6 patients refused to participate. Two patients were excluded because of hair cortisol levels greater than 1500 ng/g, leaving 44 patients available for hair analysis. This level was pre-determined to be an exclusion criterion on the basis of possible contamination from cortisol containing creams. After analysis of hair cortisol levels, there were 6 hair samples of insufficient weight for additional testosterone analysis. Therefore, 38 hair samples were available for hair testosterone analysis. Baseline characteristics of the 44 patients are presented in Table 1.

Table 1: Baseline characteristics of study participants.

	Study population (n=44)	Reference Range
Age, years	69.8 (11.2)	
NYHA class		
I/II/III, %	29/45/26	
Ischemic heart failure cause, %	84.2	
Echocardiographic features		
LVEF, %	29.4 (7.7)	
LVEDD, mm	61 (7.8)	
Comorbidities/risk factors		
BMI, kg/m²	26.4 (3.9)	
Hypercholesterolemia, %	79.2	
Diabetes mellitus, %	29	
Hypertension, %	68.4	
Current cigarette smoking, %	18.4	
Atrial fibrillation, %	23.7	
Medications		
ACE inhibitors or ARB, %	89	
β-Blockers, %	94.7	
Spironolactone, %	36.8	
Diuretics, %	57.9	
Cardiac glycoside, %	13.2	
Devices		
CRT, %	13.2	
AICD, %	44.7	
Laboratory parameters		Reference Range
Hemoglobin, mg/dl	13.3 (1.5)	13.5 - 15.5
GFR-MDRD, ml/min/1.73m²	63.6 (23.3)	>90
NT-proBNP, pg/ml	1976.8 (2957.2)	0 - 100
Hs-CRP, mg/dl	0.8 (1.0)	0 - 0.6

Values are mean (SD) or %. LVEF indicates left ventricular ejection fraction; LVEDD, left ventricular end diastolic diameter; BMI, body mass index; ACE, angiotensin converting enzyme; ARB, angiotensin 2 receptor blocker; CRT, cardiac resynchronization therapy; AICD, automatic implantable cardioverter defibrillator; GFR-MDRD, glomerular filtration rate estimated by the Modification of Diet in Renal Disease formula; NT-proBNP, N-terminal pro-brain natriuretic peptide; Hs-CRP, highly sensitive C-reactive protein.

4.3.1 Cortisol

Hair cortisol for the 44 CHF patients was 207 (117.7 - 1277.3) ng/g. Hair cortisol concentrations were positively correlated with NYHA class using Spearman's rank (r=0.48, p=0.001). Furthermore, hair cortisol levels specified for the 3 NYHA functional classes are presented in Figure 9. Hair cortisol concentrations for NYHA class 1, 2, and 3 were 159.02 (137.51 – 413.47) ng/g, 197.74 (117.68 – 1277.29) ng/g, and 363.12 (183.27 – 685.57) ng/g, respectively. Hair cortisol concentrations of NYHA stage 3 patients were significantly higher than levels in patients with stage 1 and stage 2. There was no significant difference in hair cortisol concentrations between NYHA stage 1 and stage 2 heart failure patients.
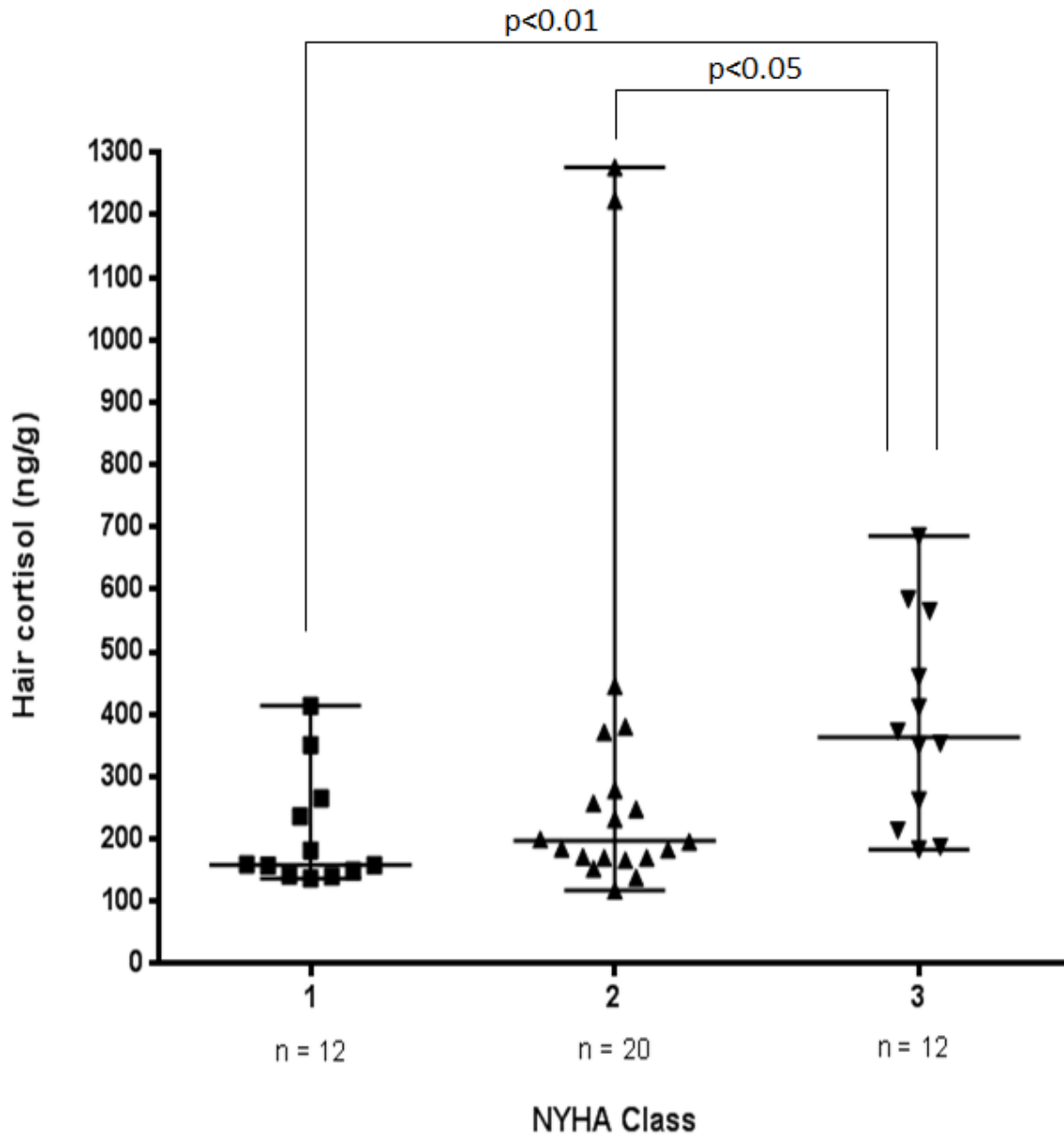
Figure 9: Hair cortisol content of CHF patients specified for NYHA stage. Median (range) hair cortisol concentrations in stage 1, 2, and 3 heart failure patients were 159.02 (137.51 – 413.47) ng/g, 197.74 (117.68 – 1277.29) ng/g, and 363.12 (183.27 – 685.57) ng/g, respectively.

NYHA class correlated as well with NT-proBNP levels (r=0.54, p<0.01), LVEF (r=-0.64, p<0.01), and exercise capacity (r=-0.80, p<0.01).

Of the 44 patients, 6 were not fit to perform a meaningful stress test due to non-cardiac causes (mainly orthopedic limitations), leaving 38 patients available for exercise capacity assessment. Hair cortisol concentrations were negatively correlated with treadmill stress test performance (r=-0.37, p<0.05) (Figure 10).

Figure 10: Exercise capacity (metabolic equivalents) and hair cortisol concentrations (ng/g).

There was no statistically significant correlation between exercise capacity and either LVEF (r=0.13, p=0.44) or NT-proBNP (r=-0.07, p=0.70). We found no significant relationship between hair cortisol concentrations and LVEF (r=0.14, p=0.37) or NT-proBNP (r=0.19, p=0.22).

During a 1-year follow-up, 25 (56.8%) patients had a CHF-related hospitalization. We found a non-significant trend toward higher hair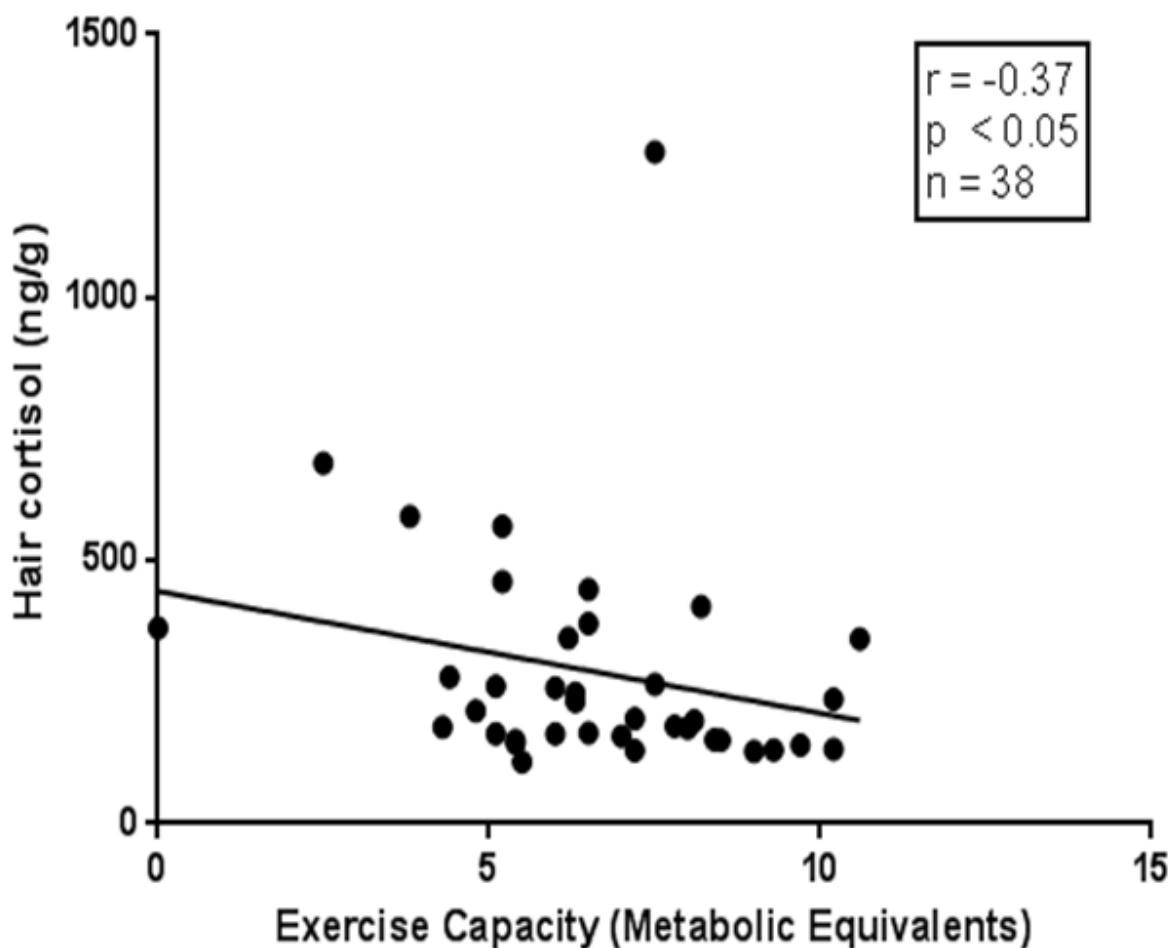 cortisol levels in the hospitalized patients as compared to non-hospitalized patients; results were 183.61 (137.51 – 1277.39) ng/g and 278.60 (117.28 – 1224.49) ng/g, respectively (p<0.08) (Figure 11A).

The odds ratio for hair cortisol levels comparing patients with a CHF-related hospitalization to those without a hospitalization was also non-significant (OR=1.00, CI [0.99-1.00]. Three patients died during the study period. This low number precluded any meaningful mortality analysis; however, hair cortisol concentrations for the patients who died were 187, 372.72, and 411.17 ng/g.

4.3.2 Testosterone

The hair testosterone content in 38 patients was 5.17 (2.39 – 24.64) ng/g. There was no statistically significant association between hair testosterone and NYHA class (r=0.23, p=0.16), LVEF (r=-0.22, p=0.18), NT-proBNP (r=0.13, p=0.43), and exercise capacity (r=-0.07, p=0.63).

Of the 38 patients, 17 had a CHF-related hospitalization at some point during the year after hair sampling. There was no difference in hair testosterone levels between hospitalized and

non-hospitalized patients; results were 5.12 (2.39 - 24.64) and 6.53 (2.40 - 15.98) ng/g, respectively (p=0.61) (Figure 11B).

4.3.3 Cortisol over Testosterone Ratio

The C/T ratio in 38 patients was 39.89 (12.98 – 173.73). There was no statistically significant correlation between the C/T ratio and NYHA class (r=0.17, p=0.30), LVEF (r=0.035, p=0.84), and NT-proBNP (r=0.13, p=0.45). The association between the C/T ratio and exercise capacity did not reach statistical significance, however we found a trend toward a negative association between C/T ratio and exercise capacity (r=-0.28, p=0.09).

The C/T ratios for non-hospitalized and hospitalized patients were 37.53 (19.64 – 76.11) and 51.63 (12.97 – 173.68), respectively (p<0.05). A comparison between patients who had a CHF-related hospitalization in the year following the inclusion in the study and patients who did not have a CHF-related hospitalization revealed a higher C/T ratio in hospitalized patients (OR = 1.04 (4%),CI [1.02 – 1.06], p<0.05) (Figure 11C). This odds ratio suggests that for every one-unit increase in the C/T ratio, the odds of hospitalization increases 4%.
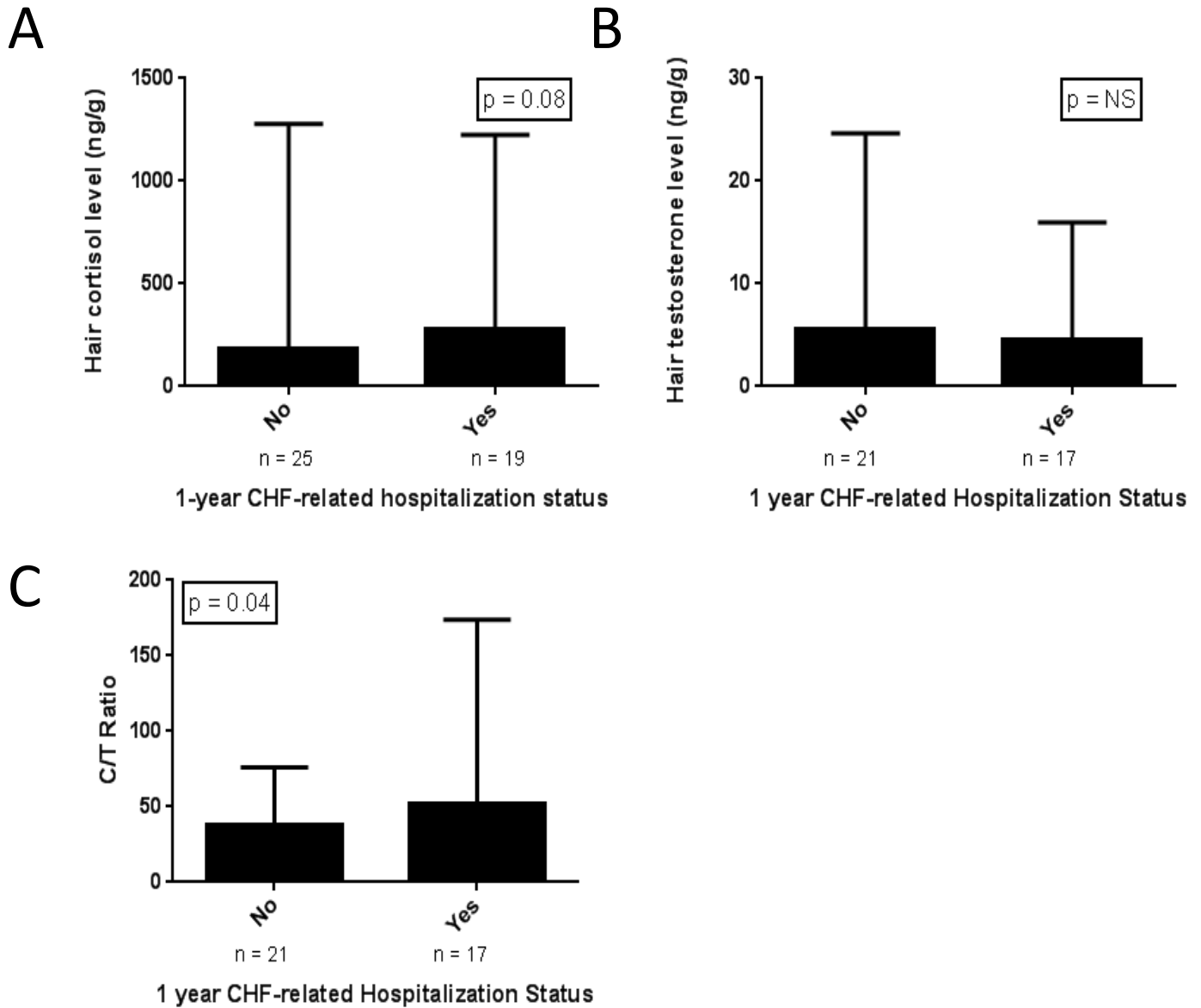
A

B

C

Figure 11: Median (range) hair cortisol concentrations for non-hospitalized and hospitalized patients (Panel A). Median (range) hair testosterone levels for non-hospitalized and hospitalized patients (Panel B). Median (range) C/T ratios of non-hospitalized and hospitalized patients (Panel C).

4.4 Discussion

The present study is the first to measure hair cortisol and testosterone as biomarkers for severity of CHF in men. We demonstrated that hair levels of cortisol correlated positively with NYHA class and negatively with exercise capacity. These findings are of clinical significance since both the NYHA score and exercise capacity have been consistently included among the most powerful predictors of morbidity and mortality in patients with CHF (Pocock et al. 2006; O'Connor et al., 2012). We did not find any statistically significant association between hair testosterone and LVEF, NYHA class, exercise capacity, NT-proBNP, and one year CHF-related hospitalizations. Similarly, we did not find any statistically significant association between the C/T ratio and LVEF, NYHA class, NT-proBNP. On the other hand, patients who had a CHF-related hospitalization had a significantly higher hair C/T ratio than patients who did not have at CHF-related hospitalization. We also demonstrated a tendency towards a negative association between C/T ratio and exercise capacity; however this did not reach statistical significance (p=0.09), possibly due to limited power.

The association of high cortisol levels with adverse outcome of patients with CHF may have several possible explanations; however from this study, it is not entirely clear if CHF causes stress resulting in increased cortisol levels, or that the increased cortisol negatively affects cardiac function. First, cortisol is known to unfavorably affect classic cardiovascular risk factors including hypertension and insulin resistance which in turn can increase the cardiovascular risk (Brunner et al., 2002). Further, we have previously demonstrated hair cortisol was increased in patients admitted with acute myocardial infarction as compared to levels in control subjects (Pereg et al., 2011), indicating that systemic cortisol exposure in the two months before

admission was higher in patients who developed an acute myocardial infarct than in those in whom this did not happen. If cortisol indeed plays a causative role in the development of CHF, then it may be possible that CHF patients with high hair cortisol levels may benefit from a more intensive assessment and treatment of the existing conventional cardiovascular risk factors, and perhaps even from interventions that would reduce cortisol levels. Secondly, it is possible that the high cortisol level itself is not a direct cause for adverse outcome but rather a marker for high risk patients.

The evidence is mixed with regard to testosterone levels and cardiovascular disease risk (Liu et al., 2003). Some studies have found an association between low testosterone and increased risk (Choong et al., 2010; Traish et al., 2011; Ullah et al., 2011), while others have not (Contoreggi et al., 1990; Arnlöv et al., 2006). A study of 208 men with heart failure found that deficiencies in serum testosterone were associated with increased mortality (Jankowska et al., 2006). On the other hand, a prospective population-based study of 2084 participants without CVD at baseline, did not find an association between serum testosterone levels and incident CVD (Arnlöv et al., 2006). Moreover, an analysis of the Framingham population did not find an association between low testosterone and cardiovascular risk (Haring et al., 2013), similar to our findings with hair testosterone. In a study by Güder et al. (2010), free testosterone was found to be inversely associated with NYHA class and NT-proBNP. We did not find any association between hair testosterone and NYHA class or NT-proBNP. The lack of association between hair testosterone and serum NT-proBNP is not surprising as hair is a measurement of average long-term hormone exposure, while measurements from serum are point measurements. As in the present study, Güder et al. also did not find an association between LVEF and free testosterone levels. Additionally, several studies have shown that testosterone supplementation improves

exercise capacity in patients with CHF (Pugh et al., 2004; Malkin et al., 2006; Caminiti et al., 2009; Iellamo et al., 2010). We hypothesize that testosterone in hair may reflect free testosterone. In a recent study, we found a trend toward correlation between hair testosterone and the Free Androgen Index, while there was no such trend for total testosterone in serum (Chan et al. unpublished observations).

To our knowledge, the present study and the Caerphilly study are the only two studies that prospectively investigate the C/T ratio in relation to CVD risk. In the Caerphilly study, a positive association was found between serum C/T ratio and incident ischemic heart disease and insulin-resistance during a mean of 16.5 years of follow-up (Smith et al., 2005). In the present study, hair C/T ratio was able to predict CHF-related hospitalization status during the following one year (OR 1.04, p=0.04). This is a potentially important result suggesting that the hair C/T ratio may be superior to hair cortisol or testosterone alone at predicting morbidity and may be used as a mode for identifying high risk heart failure patients.

Several limitations of this study warrant consideration. Our analysis pertains only to male patients. Since CHF may be differentially affected based on gender, our results should be extrapolated to females with caution. Additionally, the size of our study was relatively small and patients were recruited in a single center. Therefore, it was not possible to control for clinical variables such comorbid conditions and medications. Nevertheless, the fact that despite a relatively small sample size hair cortisol levels correlated with both the NYHA class and the exercise capacity further supports the importance of measuring hair cortisol in heart failure. Additional studies with larger sample sizes are required to compare the reliability of hair cortisol and testosterone measurement in the assessment and risk stratification of CHF patients with that of the existing clinical and laboratory parameters. Moreover, this study did not use any

psychological stress questionnaires or collect any information on hair-related variables such as colour and frequency of hair washes. It also remains to be determined whether hair cortisol and testosterone levels are predictors of the main clinical endpoints in heart failure, such as sudden cardiac death, cardiovascular death and re-hospitalization rates. Furthermore, it is not known if changes in hair cortisol and testosterone over time predict or follow changes in clinical condition and/or treatment of CHF. Equally, we do not know if measurement of morning blood samples for cortisol and testosterone over time would have been congruent with the hair results. Finally, there are limitations to the measurements of hormones in hair. Obviously, it can only be done in patients with sufficient length of hair and is potentially subject to contamination by cortisol-containing creams. Additionally, the hair growth rate is assumed to be approximately 1 cm per month in healthy individuals. Our study population consisted of elderly patients with CHF, and it is not known if either the age and/or the impaired health may affect the hair growth rate, and thus the interpretation of timing of our results. Future studies that document the hair growth rate in relation to these and other factors are warranted. Furthermore, hair cortisol cannot be used to study dynamic impacts of morning awakening response of cortisol.

In conclusion, our results suggest that higher hair cortisol levels correlate with the clinical severity of CHF as assessed by the NYHA score and treadmill exercise capacity. Going forward, our findings also highlight the potential importance of the C/T ratio measurement in hair, which may become useful as a research tool to assess other cardiovascular diseases such as myocardial infarctions. As this is the first study to use hair to measure cortisol and testosterone in patients with systolic heart failure, our results should be confirmed by future studies.

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CHAPTER 5: CONCLUSION

The objective of this research was to determine if hair cortisol, testosterone, and the C/T ratio reflect heart failure status and prognosis. This was the first study to measure hair cortisol and testosterone concentrations in CHF patients and determine any association of these hormones and their ratio with current CHF prognostic markers. We hypothesized that high hair cortisol and low hair testosterone levels serve as biological markers of poor congestive heart failure prognosis.

Hair cortisol levels were positively correlated with NYHA class and negatively associated with exercise capacity. No significant relationships between hair cortisol levels and LVEF or NT-proBNP were found. We found a non-significant trend toward higher hair cortisol concentrations in patients who were hospitalized during the year following inclusion into the study. On the other hand, there were no associations between hair testosterone and any of the current HF prognostic markers. Likewise, the C/T ratio also was not related to any of the HF markers; however a negative trend between the C/T ratio and exercise capacity was found. A comparison between patients who were hospitalized during the year following inclusion into the study and patients who were not hospitalized revealed a higher C/T ratio in patients who were hospitalized (OR=1.04). This odds ratio suggests that for every one-unit increase in the C/T ratio, the odds of hospitalization increases 4%.

Our results support our hypothesis that hair cortisol may serve as a biological marker of poor CHF prognosis. However, a limitation of using hair cortisol as a biological marker of CHF is that high hair cortisol levels are not specific to CHF. There are other reasons as to why an individual has elevated hair cortisol levels such as chronic pain and unemployment (Van Uum et al. 2008, Dettenborn et al. 2010). Our results regarding hair testosterone alone does not support our hypothesis that low testosterone serves as a biological marker of poor CHF prognosis. The results regarding the C/T ratio are particularly interesting and support our hypothesis. Additional studies are warranted to confirm our results.

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CHAPTER 6: STRENGTHS AND WEAKNESSES OF HAIR TESTING

6.1 Strengths

Cortisol and testosterone are typically measured in blood, urine, or saliva; however, these matrices only provide a window of systemic hormone exposure over a period of 24 hours or less, can be invasive, and may require repeated measurements. Scalp hair grows at a rate of 0.6 to 1.4 cm per month (Wennig et al. 2000), allowing retrospective assessment of cortisol and testosterone exposure over a period of months and years with just a single sample collection. Many reviews of the literature have supported the validity of hair cortisol as a biological marker for chronic stress and human disease (Meyer et al. 2012, Russell et al. 2012, Gow et al. 2010). Hair collection is non-invasive, does not require health care workers, and can be conducted at any time of the day. Additionally, only a small amount of hair is required (minimum 10 mg of hair) for analysis. These features make hair collection well tolerated by participants.

Another advantage of using hair as a matrix is that samples can be stored at room temperature and be sent by mail. We have demonstrated that mean hair cortisol levels from Peruvian mummies dating back to AD550 is similar to healthy volunteers today, suggesting cortisol's stability over long periods of time (Webb et al. 2011). Levels reflect average hormone levels over a period of months and years, whereas blood and saliva samples that reflect one moment in time, and urine that reflects levels during one day. Single time-point assessments using serum or urine can also be affected by the sampling itself and may provide a poor reflection of normal, long-term hormone levels and may be subject to diurnal variation (Stalder

et al. 2012). By contrast, hormone levels measured in hair are not affected by acute stress and hence may potentially be used even at or immediately after a major event, such as a myocardial infarction, which would cause acute changes in hormonal levels and thus not represent the hormonal milieu before the event (Pereg et al. 2011).

Measurements of hormones in hair, for the first time, provide an opportunity to provide a "timeline" of hormone exposure through segmental analysis. This is eloquently demonstrated by Manenschijn et al. in a study investigating if retrospective timelines of cortisol exposure could be created, using hair, in suspected cyclic Cushing's patients and assessing if the timeline corresponds with symptomatic periods (Manenschijn et al. 2012). They demonstrated that hair samples can provide a historical timeline that corresponds to the symptomatic periods in patients with cyclic Cushing's (Figure 10). It is evident that hair could be a useful diagnostic tool for early detection such a condition, versus obtaining multiple, random serum, saliva, or urine samples.
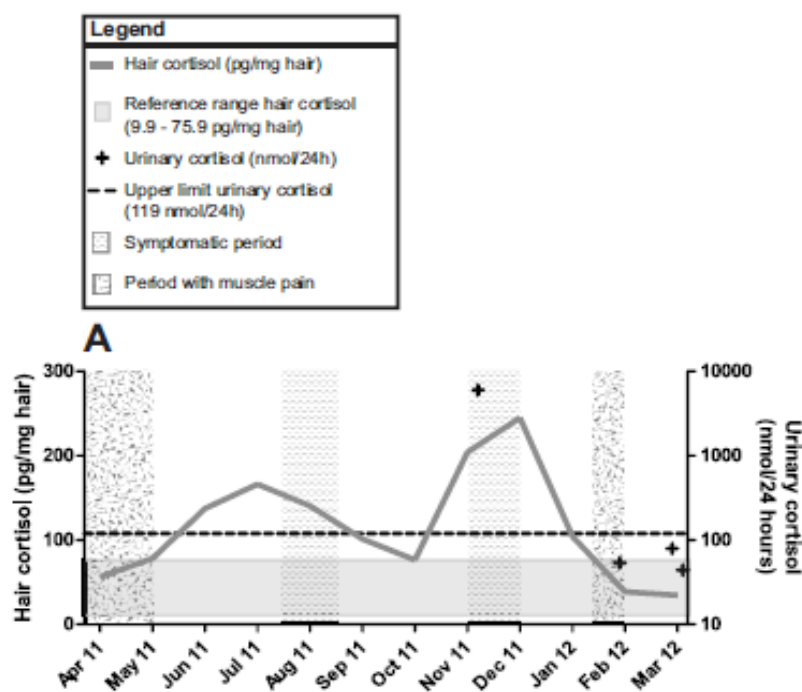

Figure 12: Hair cortisol timeline of a patient with cyclic Cushing's. (Adapted from Manenschijn et al. 2012)

6.2 Weaknesses

There are several potential limitations to measurement of endogenous hormones in hair. Hair analysis is also unable to detect acute changes in hormone levels or the impact of brief stressors. Also, clinically relevant reference ranges for hormones measured in hair are yet to be established. Additionally, hair sampling is obviously limited to individuals who have sufficient hair at the vertex posterior and do not have cultural/religious objections to taking a hair sample. Studies investigating the effect of hair dying have shown that hair dying decreases cortisol concentrations compared to controls (Sauve et al. 2007; Manenschijn et al. 2011). A proposed mechanism for this phenomenon may be that the procedure for dying hair damages the hair shaft allowing cortisol to be leached out. Another mechanism is that dye may add extra weight to hair, thus erroneously decreasing hormone concentrations after correcting for weight. No influence of natural hair colour has been reported to affect hormone concentrations in human hair (Sauve et al. 2007; Kirschbaum et al. 2009; Manenschijn et al. 2011). Finally, hair can be easily contaminated with the use of cortisol containing creams; therefore these individuals are typically excluded from studies measuring hair cortisol.

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CHAPTER 7: FUTURE DIRECTIONS

Hair is a unique tool that can be used to assess hormone exposures in the distant past (depending on the length of hair). The evidence is mixed as to whether or not hair cortisol concentrations are lower in the more distal ends of the hair shaft compared to the more proximal ends. Some studies have shown a declining pattern (Kirschbaum et al. 2009; Dettenborn et al. 2010; Gao et al. 2010; Skoluda et al. 2011), whereas others have not (Thomson et al. 2010; Manenschijn et al. 2011). A possible mechanism for this decline may be that distal hair segments are more damaged due to UV light and frequent washing. Indeed, Hamel et al. has demonstrated repeated washing of rhesus monkey hair with shampoo or water decreased hair cortisol levels (Hamel et al. 2011). However, human research has not found hair washing to effect hormone concentrations at least with respect to the proximal segments of hair (Manenschijn et al. 2011; Dettenborn et al. 2012). On the other hand, hair washing appears to lower hair cortisol content only in the distal segments (Dettenborn et al. 2012). These studies suggest that hair washing habits should be collected in future studies.

It is generally accepted that hormones measured in hair reflect systemic hormone concentrations; however, there is some evidence that supports local cortisol production by hair follicles (Sharpley et al. 2010). To what extent local production of cortisol contributes to measurements remains an area to be investigated.

Future studies should aim to take advantage of hair analysis' ability to retrospectively obtain levels of hormones prior to an event, similar to our study on patients who had a recent myocardial infarction (Pereg et al. 2011). Indeed, our group has recently collected the hair of patients who had an ischemic stroke immediately after the ischemic event to determine if chronic

stress is a risk factor for strokes (Chan et al. *in prep*). Our preliminary results of this study show that hair cortisol levels of patients who had a recent ischemic stroke were not different compared to controls.

More intervention studies would also be of interest. In 2010, our lab showed that hair cortisol concentrations decrease in patients with Cushing's syndrome after surgical removal of an adrenal mass (Thomson et al. 2010). We have also demonstrated that hair testosterone levels of hypogonadal men receiving testosterone injections were significantly higher than those not receiving treatment (Thomson et al., 2009). Importantly, hair testosterone of men treated with testosterone was not significantly different from eugonadal men. These findings suggest that testosterone can be measured in the hair as a matrix and may be useful for monitoring testosterone therapy. Similarly, patients treated with hydrocortisone in patients with adrenal insufficiency showed higher hair cortisol concentrations compared to controls, suggesting these patients may be over-treated (Gow et al. 2010).

It is certainly an exciting time for cortisol and testosterone detection in hair. The use of hair as a matrix to measure cortisol and testosterone provides a unique means of understanding the role of the long-term effects of these hormones.

7.1 References

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Curriculum Vitae

Justin Chan

Education

2012 – Present	**Master of Physiology and Pharmacology (MSc.),** Western University Thesis: "Cortisol and testosterone in hair as biological markers of systolic heart failure." Advisors: Dr. Gideon Koren MD, Dr. Michael Rieder MD, Dr. Stan Van Uum MD, PhD
2007 – 2011	**Bachelor of Medical Science (BMSc.)** *with Distinction***, Honors Specialization in Clinical Biochemistry,** Western University

Honours, Scholarships, and Awards

2013	**Best Poster Presentation Award,** 18th Scientific Meeting of the Society of Hair Testing (August 28-30), Geneva, Switzerland.
2012	**Pediatrics Graduate Studentship Award,** Schulich School of Medicine and Dentistry Awarded for strong academic track record and potential. Competition: Three awards each valued at $17,000 awarded.
2012	**Western Graduate Research Scholarship,** Western University Awarded for academic achievement ($1500)
2007 – 2011	**Queen Elizabeth II Aiming for the Top Scholarship,** OSAP Awarded for academic achievement in high school and university ($3500 renewed all years)
2007 – 2011	**Dean's Honor List with Distinction,** Western University Named for academic excellence in university
2007 – 2011	**Society of Western Scholars,** Western University Elected for academic performance in high school and university
2007	**Western Entrance Scholarship of Excellence,** Western University Awarded for academic average of 90% - 94.9% in high school ($2000)

Publications

2014	**Chan J,** Koren G, Van Uum S. Review: Cortisol and cardiovascular disease risk. *In preparation.*
2014	**Chan J,** Spence D, Rieder R, Van Uum S, Koren G. Hair cortisol in ischemic stroke patients. *In preparation.*

2014	**Chan J,** Sauvé B, Tokmakejian S, Koren G, Van Uum S. Measurement of cortisol and testosterone in hair of obese and non-obese human subjects. *Accepted for Publication in Exp Clin Endo and Diab.*
2014	**Chan J,** Natekar A, Einarson A, Koren G. Motherisk Update: Risks of Untreated Depression in Pregnancy. Can Fam Physician. Mar;60(3):242-3.
2013	**Chan J,** Natekar A, Koren G. Motherisk Update: Hot Yoga and Pregnancy; Fitness and Hyperthermia. Can Fam Physician;60(1):41-2.
2013	Pereg D*, **Chan J***, Russell E, Berlin T, Mosseri M, Seabrook J, Van Uum S, Koren G. Cortisol and testosterone in hair as biological markers of systolic heart failure. Psychoneuroendocrinology;38(12):2875-82. ***denotes equal contribution**
2013	**Chan J**, Koren G. Is Mild-Moderate Drinking in Pregnancy Harmless? New Experimental Evidence to the Opposite. J Popul Ther Clin Pharmacol;20(2):e107-e109.

Invited Talks and Conference Presentations

2014	Cortisol and testosterone in hair as biological markers of systolic heart failure, **Oral Presentation** (February 26), **Robarts Molecular Medicine Data Club,** Robarts Research Institute, London, Ontario
2013	Cortisol and testosterone in hair as biological markers of systolic heart failure, **Poster Presentation** (August 28-30), **18th Scientific Meeting of the Society of Hair Testing,** Geneva, Switzerland
2013	Hair cortisol over testosterone ratio predicts hospitalization in congestive heart failure patients, **Poster Presentation** (May 30), **Department of Medicine Research Day**, London, Ontario
2013	Hair cortisol and cardiovascular disease, **Oral Presentation** (April 17), **Endocrinology Rounds**, St. Joseph's Hospital, London, Ontario
2013	Hair cortisol levels in patients with systolic heart failure, **Poster Presentation** (March 19), **London Health Research Day**, London, Ontario
2013	Hair cortisol levels in patients with systolic heart failure, **Poster Presentation** (March 15), **Western Research Forum**, Western University, London, Ontario
2013	Hair cortisol levels in patients with systolic heart failure, **Poster Presentation** (March 5-9), **114th Annual meeting of the American Society of Clinical Pharmacology and Therapeutics**, Indianapolis, Indiana, USA

2012	Hair cortisol levels in ischemic stroke patients: study design, **Poster Presentation** (November 6), **Physiology and Pharmacology Research Day**, Western University
2011	Identification and Characterization of Histatin 1 Binding Partners in the Oral Cavity, **Poster Presentation** (January 21), **Biochemistry Research Showcase Day**, Western University

Other Research Experience

2013 – Present	**Community Noise and Health Study,** Statistics Canada Health Statistics Branch

Teaching Experience

2013	**Teaching Assistant,** Pharmacology 4460a/PhysPharm 9566: Human Toxicology, Western University
2011 – 2012	**Instructional Therapist,** Aspirations & Discoveries Behavior Consultation & Education Centre, Richmond Hill, ON