An evaluation of trimetazidine as a therapeutic intervention in a newly established ex vivo mouse model of acute heart failure

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“The Road goes ever on and on

Down from the door where it began.

Now far ahead the Road has gone,

And I must follow, if I can,

Pursuing it with eager feet,

Until it joins some larger way

Where many paths and errands meet.

And whither then? I cannot say”

— J.R.R. Tolkien, The Fellowship of the Ring

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Abstract

Introduction

Acute heart failure (AHF) is the most common primary diagnosis for hospitalized heart diseases in Africa. Although Sub-Saharan women are more prone to suffer from *de novo* AHF at a much younger age, females have historically been underrepresented in biomedical research studies. As increased fatty acid oxidation (FAO) with heart failure triggers detrimental effects within the myocardium, we hypothesized trimetazidine (TMZ) (a partial FAO inhibitor) treatment will provide cardio protection to control and diabetic mouse hearts subjected to AHF. We further hypothesized that TMZ efficacy will be influenced by different phases of the estrous cycle.

Aims

1) Establish an unique *ex vivo* AHF model using hearts isolated from *db*/*db* mice and their lean control littermates (*db*/*+*); 2) Evaluate whether FA-albumin filtering can replace the gold standard method of dialysis for perfusate preparation; 3) Assess whether we can identify the different phases namely proestrus, estrus (follicular phase), metestrus and diestrus (luteal phase) of the estrous cycle in the female mice; and 4) Evaluate TMZ as a therapeutic option in our *ex vivo* AHF model for normal and obese/diabetic mice, respectively, ascertain if there are sex-based differences, and determine whether the phases of the estrous cycle can influence cardio protection.

Methods

The Langendorff retrograde isolated heart perfusion system was employed to establish an *ex vivo* AHF model that consisted of three phases: Stabilization – Krebs-Henseleit buffer (10 mM glucose) at 100 cmH_2O (25 minutes); Critical Acute Heart Failure (CAHF) – (2.5 mM glucose, 1.2 mM palmitic acid bound to 3% bovine serum albumin [BSA]) at 20 cmH_2O (25 minutes); and Recovery Acute Heart Failure (RAHF) – (10 mM glucose, 1.2 mM palmitic acid bound to 3% BSA) at 100 cmH_2O (25 minutes). 5 µM TMZ was administered in the perfusate at either the CAHF or RAHF phase for the full duration of the respective phase. The filter versus dialysis experiments were run for 30 minutes in

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Krebs-Henseleit buffer (10 mM glucose, 1.2 mM palmitic acid bound to 3% BSA). Phases of the estrous cycle were determined by vaginal smear cytology or “wet smears” and viewed under a light microscope. Enzyme-linked immunosorbent assays (ELISA) were utilized to measure serum hormonal levels while Western blotting was employed to assess protein expression levels.

Results

Our model mimicked *de novo* AHF in the switch from stabilization to CAHF and partial recovery in the switch from CAHF to RAHF. This study established that the dialysis method for FA-BSA preparations can be substituted by a simple filtering protocol. While vaginal smear cytology confirmed acyclicity of obese females (therefore lost follicular phase), lean females exhibited normal estrous cycle phases. Commercial ELISA kits were not adequately sensitive to detect hormonal fluctuations. All groups displayed a severe decrease in function during CAHF and recovery with RAHF (vs. CAHF). Lean and obese males benefited equally from TMZ treatment administered during the RAHF phase. The lean females in the two main phases of the estrous cycle (follicular and luteal) responded in distinct ways. Here lean follicular females were the only group to respond to TMZ treatment during the CAHF phase, while lean luteal females did not respond to therapy but rather displayed an inherent cardio protection that was lost with obesity. Obese luteal females also benefited from TMZ treatment during RAHF. No changes were observed in protein expression levels of 3-keotacyl-CoA thiolase (3-KAT) nor pyruvate dehydrogenase (PDH).

Conclusion

A novel *ex vivo* mouse AHF model has successfully been established and utilized the filtering method as opposed to the gold standard dialysis method. TMZ as a therapy for AHF showed great promise in improving functional recovery of mice subjected to the AHF protocol. Sex differences were present only in lean groups where the phases of the estrous cycle influenced therapy, while obesity only affected TMZ efficacy in females. The optimization of cardiac metabolism by TMZ emerges as a novel

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and worthy therapeutic option to investigate for the treatment of AHF in normal and diabetic patients (for both genders).

Opsomming

Inleiding

Akute hartversaking (AHF) is die mees algemene primêre diagnose vir gehospitaliseerde hartsiektes in Afrika. Alhoewel vrouens van Sub-Sahara Afrika meer geneig is om te ly aan *de novo* AHF op 'n veel jonger ouderdom, is vrouens histories onderverteenwoordig in biomediese navorsingstudies. Verhoogde vetsuuroksidasie (FAO) tydens hartversaking lei tot nadelige gevolge binne die miokardium. Ons vermoed behandeling met trimetazidine (TMZ) ('n gedeeltelike FAO inhibitor) sal beskerming bied aan kontrole en diabeet muis harte onderworpe aan AHF. Ons vermoed verder dat TMZ se doeltreffendheid beïnvloed sal word deur verskillende fases van die estrussiklus.

Doelwitte

1) Stel 'n unieke *ex vivo* AHF model op met behulp van harte geïsoleer vanaf *db/db* muise en hul skraal kontrole werpselmaats (*db/+*); 2) Evalueer of die filtrering van vetsuur-albumien die standaard metode van dialise vir perfusaat voorbereiding kan vervang; 3) Assesseer of ons die verskillende fases naamlik proestrus, estrus (follikulêre fase), metestrus en diestrus (luteale fase) van die estrussiklus in die vroulike muise kan identifiseer; en 4) Evalueer TMZ as 'n terapeutiese opsie in ons *ex vivo* AHF model vir normale en oorgewig/diabeet muise, onderskeidelik, stel vas of daar geslag-gebaseerde verskille teenwoordig is, en bepaal of die fases van die estrussiklus kardiale beskerming kan beïnvloed.

Metodes

Die Langendorff retrograde geïsoleerde hartperfusie stelsel is gebruik om 'n *ex vivo* AHF model te vestig wat bestaan uit drie fases: Stabilisasie – Krebs-Henseleit buffer (10 mM glukose) by 100 cmH_2O (25 minute); Kritieke Akute Hartversaking (CAHF) – (2,5 mM glukose, 1,2 mM palmitiensuur

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gebind aan 3% van bees serum albumien [BSA]) by 20 cmH_2O (25 minute); en Herstellende Akute Hartversaking (RAHF) - (10 mM glukose, 1,2 mM palmitiensuur gebind tot 3% BSA) by 100 cmH_2O (25 minute). TMZ (5 µM) is toegedien in die perfusate óf by die CAHF of RAHF fase vir die volle duur van die onderskeie fase. Die filtrering teenoor dialise eksperimente is uitgevoer vir 30 minute met Krebs-Henseleit buffer (10 mM glukose, 1,2 mM palmitiensuur gebind tot 3% BSA). Fases van die estrussiklus was bepaal deur vaginale smeer sitologie of "nat smere" en besigtiging onder 'n ligmikroskoop. Ensiem-gekoppelde immunosorberende toetse (ELISAs) is gebruik om hormonale vlakke in serum te meet, terwyl Westerse klad analises gebruik is om vlakke van proteïen uitdrukking te evalueer.

Resultate

Ons model boots *de novo* AHF na in die oorskakeling van die stabilisering fase na CAHF en gedeeltelike herstelling in die oorskakeling van CAHF na RAHF. Hierdie studie het vasgestel dat die dialise metode vir die vetsuur-BSA voorbereidings vervang kan word deur 'n eenvoudige filtrasie protokol. Vaginale smeer sitologie het bevestig dat oorgewig wyfies nie normaal deur die estrussiklus sirkuleer nie, terwyl skraal wyfies wel normaal deur die fases van die siklus beweeg. Kommersiële ELISA toetse se sensitiwiteit was nie voldoende om hormonale afwykings vas te stel nie. Alle groepe het 'n ernstige afname in funksionaliteit getoon tydens CAHF en herstel met RAHF (teenoor CAHF). Skraal en oorgewig mannetjies het ewe veel voordeel getrek uit TMZ behandeling, toegedien tydens die RAHF fase. Die skraal wyfies in die twee hoof-fases van die estrussiklus (follikulêre en luteale fases) het op verskillende maniere gereageer. Hier was die skraal follikulêre wyfies die enigste groep om te reageer op die TMZ behandeling tydens die CAHF fase, terwyl skraal luteale wyfies nie reageer het op behandeling nie, maar eerder 'n inherente kardiale beskerming getoon het wat afwesig was met vetsug. Oorgewig luteale wyfies het ook voordeel getrek uit TMZ behandeling tydens RAHF. Geen veranderinge is waargeneem in proteïen uitdrukking vlakke van 3-keotacyl-KoA thiolase (3-KAT) en piruvaatdehidrogenasekompleks (PDH) nie.

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Slot

'n Nuwe *ex vivo* muis AHF model is suksesvol gevestig en die filtrasie metode was ingestel in teenstelling met die goue standaard dialise metode. As terapie teen AHF, is TMZ heel belowend in die verbetering van funksionele herstel van muise onderworpe aan die AHF protokol. Geslagsverskille is slegs waargeneem in skraal groepe waar die fases van die estrussiklus terapie beïnvloed het, terwyl TMZ doeltreffendheid slegs in wyfies deur vetsug affekteer is. Die optimisering van hart metabolisme deur TMZ terapie kom na vore as 'n nuwe en waardige terapeutiese opsie om te ondersoek vir behandeling van AHF in normale en diabetiese pasiënte (van beide geslagte).

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List of conferences

- The International Cell Death Society meeting (May 2014). Protea hotel, Stellenbosch. Attended.

- Physiological Society of South Africa (September 2015). Khaya iBhubesi, Parys. Runner up in the Johnny van der Walt poster prize competition.

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List of abbreviations

$(dp/dt)_{max}$	Index of myocardial contraction velocity
3-KAT	3-keotacyl-CoA thiolase
ACC	Acetyl-CoA carboxylase
ACE	Angiotensin converting enzyme
ADCHF	Acute decompensating of chronic heart failure
ADHERE	Acute Decompensated Heart Failure National Registry
ADHF	Acute decompensated heart failure
ADP	Adenosine 5’-diphosphate
AGEs	Advanced glycation end products
AHA	American Heart Association
AHF	Acute heart failure
AII	Angiotensin II
AKT-1	Protein kinase B
AMP	Adenosine 3', 5'-monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocase
ATP	Adenosine 5’-triphosphate
AVP	Arginine vasopressin
BAD	B-cell lymphoma-2-associated death promoter
Bcl-2	B-cell lymphoma-2
BMI	Body mass index
BNP	B-type natriuretic peptide
bpm	Beats per minute
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
Ca^{2+}	Calcium
$CaCl_2.2H_2O$	Calcium chloride
CAD	Coronary artery disease
CAHF	Critical acute heart failure
CaMK	Calcium-calmodulin dependent kinase

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CAT	Carnitine acetyl transferase
CHF	Chronic heart failure
Co.	Corporation
CO_2	Carbon dioxide
CoQ	Coenzyme Q or ubichinone
COX-2	Cyclo-oxygenase- (COX) 2
CPAP	Continuous positive airway pressure
CPT-1	Palmitoyltransferase- (CPT) 1
CPT-2	Palmitoyltransferase- (CPT) 2
Cr/PCr	Creatine/phosphocreatine
CRP	C-reactive protein
CT	Acylcarnitine translocase
CTP	Citrate transport protein
CVD	Cardiovascular disease
CyC	Cytochrome c
CYP3A	Cytochrome P4503A
DAG	Diacylglycerol
DCM	Diabetic cardiomyopathy
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DNV	Distended neck veins
ECG	Electrocardiography
ECL	Enhanced Chemi-Luminescence
EDTA	Ethylenediaminetetraacetic acid
EHFS II	EuroHeart Failure Survey II
ELISA	Enzyme-linked immunosorbent assays
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK-1/2	Extracellular signal-regulated kinase- (ERK) 1/2
ESC	European Society of Cardiology
ET-1	Endothelin- (ET) 1
ETC	Electron transport chain
F-6-P	Fructose-6-phosphate

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FA^-	Fatty acid anions
FABP	Fatty acid-binding proteins
FACS	Fatty acyl-CoA synthase
$FADH_2$	1, 5-dihydroflavin adenine dinucleotide
FAO	Fatty acid β-oxidation
FAs	Fatty acids
FATP	Fatty acid transporter protein
FAU	Fatty acid uptake
G-3-P	Glyceraldehyde-3-phosphate
G-6-P	Glucose-6-phosphate
GC–MS/MS	Gas chromatography–tandem mass spectrometry
GAPDH	Glyceraldehyde phosphate dehydrogenase
GIK	Glucose-insulin-potassium
GLUT-1	Glucose transporter- (GLUT) 1
GLUT-4	Glucose transporter- (GLUT) 4
GO	Glucose oxidation
GSH	Glutathione
GSK-3β	Glycogen synthases kinase- (GSK) 3β
GU	Glucose uptake
H^+	Proton/hydrogen ion
H_20	Water
HBP	Hexosamine biosynthetic pathway
HDL	High-density lipoproteins
HF	Heart failure
ICAM-1	Intercellular adhesion molecule- (ICAM) 1
IL-1	Interleukin- (IL) 1
IL-10	Interleukin- (IL) 10
IL-18	Interleukin- (IL) 18
IL-1β	Interleukin- (IL) 1β
IL-6	Interleukin- (IL) 6
Inc.	Incorporated
iNOS	Inducible nitric oxide synthase
IRS-1	Insulin receptor substrate- (IRS) 1

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JNK	C-Jun N-terminal kinase
K^+	Potassium
K_{ATP}	ATP-sensitive potassium channel
KCl	Potassium chloride
KIM-1	Kidney injury molecule- (KIM) 1
LAP	Left atrial pressure
LC–MS/MS	Liquid chromatography–tandem mass spectrometry
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
L-FABP	L-type fatty acid-binding protein
LLC.	Limited Liability Company
LPL	Lipoprotein lipase
Ltd.	Limited
LVDevP	Left ventricular developed pressure
LVEDP	Left ventricular end diastolic pressure
LVEDP	Left ventricular end diastolic pressure
LVESP	Left ventricular end systolic pressure
MAPK	Mitogen-activated protein kinase
MCD	Malonyl-CoA decarboxylase
MCT	Monocarboxylate transporter
$MgCl_2.6H_2O$	Magnesium chloride
MIM	Mitochondrial inner membrane
MOM	Mitochondrial outer membrane
MPTP	Mitochondrial permeability transition pore
MR-proADM	Adrenomedullin
MTE	Mitochondrial thioseterase
mTOR	Mammalian target of rapamycin
Na^+	Sodium
Na_2CO_3	Sodium carbonate
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NADH	Nicotinamide adenine nucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

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NaF	Sodium fluoride
NaH_2PO_4	Monosodium phosphate
$NaHCO_3$	Sodium bicarbonate
NCX	Na^+/Ca^{2+}exchanger
NF-kB	Nuclear factor -kB
NGAL	Neutrophil gelatinase-associated lipocalin
NIPPV	Non-invasive positive pressure ventilation
NO	Nitric oxide
NOGPs	Non-oxidative glucose pathways
NOS	Nitric oxide synthase
NP-40	Tergitol-type NP-40
NT-proBNP	N-terminal of the prohormone brain natriuretic peptide
NYHA	New York Heart Association
O_2	Oxygen
OS	Oxidative stress
PAI-1	Plasminogen activator inhibitor- (PAI) 1
PAP	Pulmonary arterial pressure
PARP	Poly (ADP ribose) polymerase
PCr/ATP	Phosphocreatine/ATP
PDBP	Pulmonary diastolic blood pressure
PDH	Pyruvate dehydrogenase
PDHK	Pyruvate dehydrogenase kinase
PDHP	Pyruvate dehydrogenase phosphatase
PFK-1	Phosphofructokinase- (PFK) 1
PI3-K	Phosphatidylinositol 3- (PI3) kinase
PKA	Protein kinase A
PKC	Protein kinase C
plc.	Public Limited Company
PMSF	Phenylmethylsulfonyl fluoride
PPAR-α	Peroxisome proliferator activated receptor- (PPAR) alpha
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PVDF	Polyvinylidene fluoride
RAAS	Renin-angiotensin-aldosterone-system

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RAGE	Receptor for advanced glycation end products
RAHF	Recovery acute heart failure
RIPA	RadioImmunoPrecipitAtion
RNS	Reactive nitrogen species
ROS	Radical/reactive oxygen species
RPP	Rate pressure product
RyR	Ryanodine receptors
S6K	S6 kinase
SBP	Systolic blood pressure
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate- (SDS) polyacrylamide gel electrophoresis- (PAGE)
SEM	Standard error of the mean
SERCA-2	Sarco(endo)plasmic reticulum calcium-ATPase- (SERCA) 2
$SO_2^{\cdot -}$	Superoxide
SOCS-3	Suppressor of cytokine signaling- (SOCS) 3
SpO_2	Arterial oxygen saturation
SR	Sarcoplasmic reticulum
ST2	Suppression of tumorigenicity
TAG	Triacylglycerol
TBS-T	Tris-Buffered Saline -Tween 20
TCA	Tricarboxylic acid
TCE	Trichloroethanol
TEMED	Tetramethylethylenediamine
TG	Triglycerides
THESUS-HF	The Sub-Saharan Africa Survey of Heart Failure
TMZ	Trimetazidine
TNF-α	Tumor necrosis factor- (TNF) alpha
Tris-HCl	Tris-hydrogen chloride
TSH	Thyroid stimulating hormone
UA/CR	Urinary albumin/creatinine ratio
UCPs	Uncoupling proteins
VCAM-1	Vascular cell adhesion molecule- (VCAM) 1

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VEGF	Vascular endothelial growth factor
VLDL	Very-low-density lipoprotein
β-TP	β-trace protein
$\Delta\mu_H^+$	Change in electrochemical gradient

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List of measurements

˚C	Degree Celsius
<	Less-than
>	Bigger-than
≤	Less than or equal to
≥	Bigger than or equal to
µg	Microgram
µg/ml	Microgram per milliliter
µl	Microliter
µm	Micrometer
½	One half
¾	Three quarters
au	Arbitrary unit
cm	Centimeters
cmH_2O	Centimeter of water
g	Gram
kg	Kilogram per square meter
kg/m^2	Kilogram per square meter
L	Liter
m^2	Square meter
mA	Milliampere
mg	Milligram
mg/kg	Milligram per kilogram
ml	milliliter
ml/kg/h	milliliter per kilogram per hour
mm	millimeter
mM/L	Millimol per liter
mmHg	Millimeter of mercury
ng/ml	Nanogram per milliliter
pg/ml	Picogram per milliliter
rpm	Revolutions per minute
V	Volt

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v/v	Volume/volume percent
w/v	Mass/volume percent
x g	Times earth's gravitational force

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Chapter 1: Introduction

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1.1 The Grim Reaper's calling card

Cardiovascular diseases (CVD) remain the leading cause of global mortality accounting for 29.6% of all deaths. In Europe, this statistic rises to an average 45%, where in women 49% (40% of men) succumb to CVD (Townsend *et al*., 2015). According to the American Heart Association (AHA), heart failure (HF) affects 5.1 million individuals in the United States (>20 years old) and this is expected to increase by 25% by 2030 (Go *et al.*, 2013). Moreover, the socio-economic burden of CVD progressively expands with the increasing incidence of diabetes and obesity as a result of lifestyle choices and poor control of CVD risk factors (Atella *et al.*, 2009).

1.2 Defining matters of the (failing) heart

HF is a clinical syndrome that is characterized by several symptoms that lead to intolerance to effort as well as fluid retention as a result of an increased neurohumoral response due to cardiac dysfunction. Thus the pathophysiological condition known as 'heart failure' can be defined as a state where the heart does not pump sufficient blood to meet the metabolic demands of the body (Chawla & Rajput, 2012).

The lesser known and "sister" of HF, acute heart failure (AHF), poses challenges in terms of a straightforward definition and classification as it is a complex clinical syndrome that varies extensively in terms of underlying pathophysiologic mechanisms and clinical presentations (Metra *et al.*, 2010). AHF is defined by the European Society of Cardiology (ESC) guidelines as "a rapid onset or change in the signs and symptoms of HF, with accompanying raised natriuretic peptide levels and the resulting need for urgent therapy" (Mebazaa *et al.*, 2015). AHF can also be defined in terms of a patient's signs, presentation and duration of symptoms. Here a patient is placed into one of the two main groups that constitute the bigger AHF umbrella, with the first being acute *de novo* HF where patients have no pre-existing HF and this is therefore the initial manifestation of HF symptoms. By contrast, the second group or acute decompensated HF (ADHF) represents a condition where pre-existing HF is rapidly deteriorating. Only once the complexity of the underlying pathology is

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understood can the two conditions be distinguished. ADHF can also be further divided into *de novo* HF and acute decompensation of chronic HF (ADCHF) (Metra *et al.*, 2010). To further add to such complexity, acute *de novo* HF, advanced refractory HF and ADHF also fall under the umbrella term 'Acute Heart Failure Syndrome' (AHFS) (De Luca *et al.*, 2007). Multiple AHFS schemes, each containing their own classifications and cut-off points have been proposed and utilized, while others opt to employ combined schemes (Dickstein *et al.*, 2008; Gheorghiade *et al.*, 2005; Gheorghiade & Pang, 2009; Jessup *et al.*, 2009; Pang *et al.,* 2010). The term AHFS has recently been accepted by Europe, France and the United States to include five separate yet overlapping classifications and presentations (Laribi *et al.*, 2012) (Table 1.1).

AHFS – five clinical classifications and scenarios of AHF	
Hypertensive AHF	• SBP high (often > 150 mmHg) • Rales on auscultation • SpO_2 in room air < 90% • Preserved systolic ventricular function • Chest radiograph consistent with PE
Chronic decompensated HF	• History of hospitalization for a similar episode • No signs of hypertensive crisis or cardiogenic shock
Cardiogenic shock	• Low cardiac output • Low blood pressure: SBP < 90 mmHg • Oliguria < 0.5 ml/kg/h • Heart rate > 60/min
Right HF	• Predominant right signs: jugular distension • Organ congestion: liver, kidney
HF and ACS	• Rales and/or signs of low flow following a myocardial infarction

Table 1.1: Acute Heart Failure Syndrome (AHFS). Recently, three large epidemiological studies in Europe (EuroHeart survey), France (EFICA) and the United States (ADHERE) accepted this syndrome to consist of five classifications of which the underlying pathophysiology is separate, yet overlapping. AHF: acute heart failure, AHFS: acute heart failure syndrome, HF: heart failure, SBP: systolic blood pressure, PE: acute pulmonary edema, ACS: acute coronary syndrome, SpO_2: arterial oxygen saturation. Adapted from Laribi *et al.*,2012.

Thus it is abundantly clear that no distinct definition is currently available for AHF, therefore there is limited uniformity within the clinical setting and also in terms of research endeavors in this field.

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1.3 Acute heart failure: the burden of disease

AHFS that results in hospitalization is the most commonly diagnosis-related group in Medicare patients in the United States and is also the most expensive (Fang *et al*., 2008; Hunt *et al.*, 2005; Rosamond *et al.*, 2008). Patients usually seek medical help for congestion and fluid overload and not low cardiac output (Adams Jr. *et al.*, 2005; Gheorghiade *et al*., 2006). Metra *et al.* (2010) demonstrated that AHF has a 3-8% in-hospital mortality rate, a 9-13% 60-90 day mortality rate and a 25-30% short term re-hospitalization rate (Metra *et al.*, 2010). As re-hospitalizations within 1 year are reported to reach 50%, this demonstrates the severity of the disease (Bueno *et al.*, 2010). Post-discharge events could be due to renal and neurohormonal abnormalities and deterioration of general signs and symptoms of HF (Gheorghiade & Pang, 2009).

The different types of AHF display varying severities, hospital readmissions, prognosis and mortality rates. For example, ADHF has higher hospital readmission and mortality rates and this was ascribed to the presence of multiple comorbidities (Adams Jr. *et al.*, 2005). In addition, patients with acute *de novo* HF exhibited increased risk of death compared to ADHF, even though these patients were younger and displayed less co-morbidities (Follath *et al.*, 2011; Nieminen *et al.*, 2006; Tavazzi *et al.*, 2006). Such patients presented with lower blood pressure despite displaying higher left ventricular ejection fractions (Follath *et al.*, 2011). Although evidence-based therapies for HF have been initiated earlier during hospitalization, the mortality, post-discharge and re-hospitalizations remain high. Thus such findings clearly demonstrate that this is an undeniable social burden, especially when considering the number of individuals affected by various AHF conditions and the resultant costs (Pang *et al.*, 2010).

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Bringing it closer to home:

Major reviews on the pathogenesis, epidemiology and prognosis of HF and cardiomyopathy in Africans were recently published that provide useful insight in this regard (Mayosi, 2007; Ntusi & Mayosi, 2009; Sliwa *et al.,* 2005). For example, ADHF is the most common primary diagnosis in patients with heart disease admitted to African hospitals (Damasceno *et al*., 2007; Sliwa *et al.*, 2008). Moreover, while AHF is a disease of the elderly (mean age 70-72 years) in western countries (Adams Jr. *et al.*, 2005; Nieminen *et al.*, 2006), the situation is quite different for Africa where it strikes at a mean age of 52 years (Ogah *et al.*, 2015; Sliwa & Mayosi, 2013). Registries of higher income countries also reveal that women with AHFS are generally older than men (Fonarow *et al.*, 2009; Galvao *et al.*, 2006; Nieminen *et al.*, 2008; Tsuchihashi-Makaya *et al.*, 2011).However, Sub-Saharan African women are on average younger and more prone to *de novo* AHF compared to men (Ogah *et al.*, 2015). The substantial difference in AHF prevalence in young persons (20-29 year old age group) is also alarming (Figure 1.1). Cardiomyopathies in Africa pose a particular threat as it imposes on individuals already plagued by famine and other diseases. Furthermore, the resource-poor environment lacks specialized equipment needed for accurate diagnosis and limited, if any, accessibility to potentially lifesaving interventions are available. AHF therefore strikes the African homes of young mothers and breadwinners, thus further disrupting already dire household situations (Sliwa & Mayosi, 2013).

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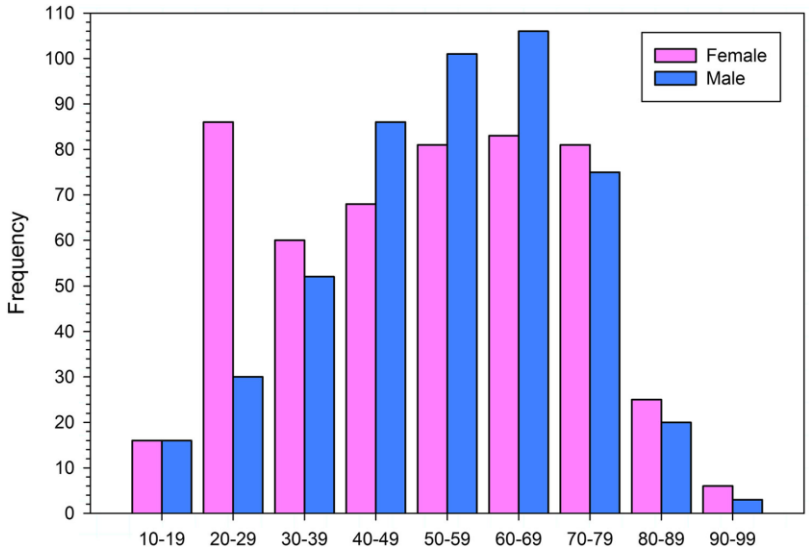

Figure 1.1: Acute heart failure (AHF) prevalence in Sub-Saharan Africa by age distribution. Females are on average significantly younger (~50.7 years old) than males (~54 years old) and a profound sex difference exists for the 20-29 year old age group (Ogah *et al.*, 2015).

Major differences are also evident in terms of ADHF epidemiological data (Sliwa & Mayosi, 2013), i.e. between the Sub-Saharan "The Sub-Saharan Africa Survey of Heart Failure" (THESUS-HF) registry (Damasceno *et al.*, 2012), the North-American "Acute Decompensated Heart Failure National Registry" (ADHERE) registry (Adams Jr. *et al.*, 2005) and the European "EuroHeart Failure Survey II" (EHFS II) registry (Nieminen *et al.*, 2006) (Table 1.2).

Characteristic	ADHERE registry (n=105,388)	EHFS II survey (n=3,580)	THESUS-HF registry (n=1,006)
Male (%)	48	61	49
Mean age (years)	72	70	52
History of HF (%)	75	63	22*
Hypertension (%)	73	63	45
CAD (%)	57	54	7
Diabetes (%)	44	33	11
Renal insufficiency (%)	30	17	8

Table 1.2: Features of patients with acute heart failure (AHF) in the ADHERE (USA), EHFS (Europe) and THESUS-HF (Sub-Saharan Africa). HF: heart failure, CAD: coronary artery disease, ADHERE: Acute Decompensated Heart Failure National Registry, EHFS: EuroHeart Failure Survey, THESUS-HF: The Sub-Saharan Africa Survey of Heart Failure registry. * In the last 12 months. Table adapted from Sliwa & Mayosi, 2013.

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While keeping the patient characteristics in mind (age difference, etiology) the in-hospital mortality (4%) and 6 months post-discharge mortality (18%) for the THESUS-HF registry was similar to European and North-American registries. This supports the assumption that once AHF strikes there is no bias in terms of individual patient characteristics (Damasceno *et al.*, 2012; Sliwa & Mayosi, 2013).

This section highlighted that African women suffering from AHFS are younger (with an exceptional stress on the 20-29 year old group) and are more prone to suffer from acute *de novo* HF. This AHF type is associated with an increased risk of death with less co-morbidities. Before this review examines AHF etiology in more detail, the under-representation of women in such studies will be discussed as the focus of this thesis is on sex differences.

1.4 Unheard voices from the hearts of women

The role of female-specific risk factors for CVD were emphasized in an AHA statement 'Cardiovascular disease in Women' in 1997 (Mosca *et al.*, 1997). These included arterial hypertension, diabetes mellitus, dyslipidemia and obesity. It also stressed that women (sometimes unlike men) exhibit different frequencies and decreases in risk factors (due to interventions). Subsequently, in 1999 the AHA and American College of Cardiology released a scientific statement on the first guidelines on the prevention of cardiac disease in women (Mosca *et al.*, 1999). This was a turning point as the scientific community increased the focus on risk factors in women. For example, Olmsted County data (1979-2000) revealed that the survival rate of HF was lower in women (Braunstein *et al.*, 2003), while less than a third was aware that CVD is the leading cause of death in this group. This reflected the dire lack of education in women. When this figure increased to 46% in 2003 (Mosca *et al.*, 2004), multiple campaigns were launched in an effort to promote the awareness of CVD affecting women. For example, in 2004 the seminal work of Mosca ('Evidence-Based Guidelines for Cardiovascular Disease Prevention in Women') was published (Mosca *et al.*, 2004). This was followed by the AHA's 'Go Red for Women', the ESC's 'Women at Heart' and the Spanish Society of Cardiology's 'Cardiovascular Disease in Women Working Group' campaigns (Alfonso *et al.,*

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2006; Kolovou *et al*., 2011). Moreover, clinical guidelines for the prevention of CVD in women were updated in 2007 (Mosca *et al.*, 2007). These efforts therefore reflect an increased emphasis to better understand female-specific CVD, in stark contrast to previous research where this option was largely ignored.

The CVD risk of females has been grossly undervalued in the past due to the misperception that women are protected through estrogen (Healy, 1991). However, epidemiological data showed that CVD burden is progressively expanding due to increasing diabetes and obesity and that HF is expected to increase by 25% by 2030 (Santulli, 2013). Although HF in women contributed to 35% of the total CVD mortality (Koelling *et al.,* 2004; Rathore *et al.*, 2003; Roger *et al.*, 2011), this group has historically been under-represented in HF clinical trials. For example, past clinical trials enrolled only 17%-23% women participants (Heiat *et al.* 2002). This has now been rectified and recent large scale studies recruit near equal numbers of males and females. Unfortunately, previous under-representation in clinical trials has hampered incorporation of guidelines for the treatment of female-related CVD. For example, the 2008 US guidelines on CVD (Melloni *et al.*, 2010; Mosca *et al.*, 2007) are primarily based on research performed on males, while the 2008 ESC guidelines barely addressed gender issues (Dickstein *et al.*, 2008; Maas *et al.*, 2011).

HF signs and symptoms in women can be difficult to interpret and this can lead to misdiagnosis. Moreover, among patients hospitalized for HF, women are more likely to have extended stays (Klein *et al.*, 2011). Although the ADHERE registry reported no sex differences in terms of in-hospital mortality, significant differences were noticed in treatment. Here women received less evidence-based therapies and often did not receive potentially lifesaving invasive testing or procedures as was the case for men. This trend was continued in the AHA 'Get With The Guidelines-Heart Failure' registry (Galvao *et al.*, 2006; Hernandez *et al.*, 2007; Shin *et al.,* 2012). However, women who did receive therapies were usually prescribed suboptimal doses (Klein *et al.*, 2011).

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As discussed, the lack of uniformity regarding the definition of AHF has led to contradicting data especially for mortality and survival. For example, some studies established that women have a better survival rate (Adams Jr. *et al.*, 1999; Deswal & Bozkurt, 2006; Ghali *et al.*, 2003; Levy *et al.*, 2002; Martinez-Sellés *et al.*, 2003; O'Meara *et al.*, 2007; Rathore *et al.*, 2003; Roger *et al.*, 2004; Simon *et al.,* 2001; Taylor *et al.*, 2006), while others reported mortality rates comparable to those for men (Al Suwaidi *et al.*, 2012; De Maria *et al.*, 1993; Galvao *et al.*, 2006; Nieminen *et al.*, 2008; Opasich *et al.*, 2000; Opasich *et al.*, 2004).

Even though women have less CVD risk in pre-menopause compared to age-matched men, this difference is no longer evident five years post-menopause (Harman, 2006; Hayward *et al.,* 2000). In addition, although chronic heart failure (CHF) incidence (Ho *et al.,* 1993) is lower in women, the risk of mortality is higher (Johnson, 1994; Wittnich *et al.,* 2013). Thus the concept of the "double edged sword" emerges, i.e. although women appear to be "protected" against CVD, they tend to fare far worse if an insult actually does occur.

It becomes clear then that past under representation of women has resulted in a significant effect on the forward momentum of scientific advancement. It is therefore crucial that researchers include both sexes (whether it is clinical or animal studies) in planned research studies. The emphasis will now shift to a more comprehensive review of AHF etiology and presentation. A general review of this condition will initially be covered and thereafter the attention will shift to AHF in women as this is the focus of this laboratory-based study.

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1.5 The sources, co-conspirators and presentation of the failing heart

Co-morbidities may act as an underlying disease(s) or it may serve as a trigger for AHF. It not only influences clinical manifestations and prognosis, but also contributes to pathology. Five categories of AHF etiology can be distinguished: (1) acute coronary syndrome, cardiomyopathies, hypertension and different arrhythmias; (2) Iatrogenic etiology, consisting of therapies such as cardio-toxic chemotherapy, beta blockers and calcium (Ca^{2+}) channel blockers; (3) metabolic etiology such as diabetes mellitus and thyroid abnormalities; (4) infiltration, that includes among others, amyloidosis and sarcoidosis and (5) other that can include a wide variety including diet, valvular disease and peripartum cardiomyopathy (Laribi *et al.*, 2012).

According to the ADHERE and EHFS II registries, the large epidemiological contributions were coronary artery disease (CAD), hypertension and diabetes (Adams Jr. *et al.*, 2005; Nieminen *et al.*, 2006). For another study comparing European and US data, the contribution of hypertension and diabetes for all AHF individuals was 59.4% and 44.6%, respectively (Karasek *et al*., 2012). As underlying disease and trigger events, CAD contributed 71% and 36.1% respectively, while hypertension contributed 11% and 21.3%, respectively. This mimics the African context where AHF affects younger patients and factors most likely to contribute would include hypertension, diabetes and obesity. Interestingly, ischemic heart disease has the lowest etiology contribution within the African context (Damasceno *et al.*, 2012; Sliwa & Mayosi, 2013).

Data from registries (developing world) show that women with ADHF tend to be older, present with more severe HF and display increasingly frequent signs and symptoms of HF, lower quality of life and worse impairment of functional capacity (Deswal & Bozkurt, 2006; Galvao *et al.*, 2006; Koelling *et al.*, 2004; Levy *et al.*, 2002; Roger *et al.*, 2004). This therefore demonstrates the importance of investigating AHF in women.

There are remarkable differences in AHF-related symptoms between men and women. Women are more likely to present with preserved ejection fraction as a result of hypertension and diabetes.

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They also present with higher systolic blood pressure and ejection fraction on admission. In addition, they are less likely to present with CAD and ischemic etiology. By contrast, men are usually diagnosed with systolic HF as a result of ischemia and previous myocardial infarctions. Thus men are also more likely to be burdened with CAD and impaired left ventricular function (Adams Jr. *et al.*, 2005; Al Suwaidi *et al.*, 2012; Klein *et al.*, 2011; Nieminen *et al.*, 2008; O'Meara *et al.*, 2007; Owan *et al.*, 2006; Regitz-Zagrosek *et al.,* 2007). Of note, increased total cholesterol levels are found in female AHF patients of all ages (Spinarova *et al.*, 2012). Moreover, there are also differences in terms of left ventricular remodeling where women display more severe hypertrophy in smaller hearts (an intrinsic sex difference), while the men exhibit more dilation and fibrosis due to pressure overloads (Fliegner *et al.*, 2010; Petrov *et al.*, 2010). As these results demonstrate, AHF has distinct characteristics in men and women and it is therefore shortsighted to simply extrapolate the results of male-based studies to females.

1.5.1 Diabetes

Diabetes can be present in up to 35%-40% of AHF cases. However, with right ventricular AHF it is present in only 24.5% of cases (Spinarova *et al.*, 2012). Co-morbidities-and risk factors do not completely explain the compelling and independent role that diabetes plays as a CVD risk factor (De Simone *et al.*, 2010; Horwich & Fonarow, 2010). For example, diabetes has an independent association with death (from any cause) for HF patients (Martínez-Sellés *et al.*, 2012). Moreover, despite intensive treatment regimens diabetic AHF patients possess a higher in-hospital mortality rate than their non-diabetic counterparts (Parissis *et al.*, 2012). Type 2 diabetes is a stronger risk factor for CVD in women than in men (Becker *et al.*, 2003) and here females display a higher mean systolic blood pressure and increased cholesterol and hypertension are also more frequent. Their cardiovascular risk profile is worse and evidence shows that they reach therapeutic goals less often (Kautzky-Willer *et al.*, 2010). In agreement, fatal ischemic heart disease risk is 50% higher in female type 2 diabetic persons (Huxley *et al.,* 2006), while the Framingham study revealed that HF was several times higher in the diabetic women versus male counterparts (Kannel *et al.*, 1974). To

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summarize, diabetes is a particularly strong and deadly risk factor for HF in women (Bibbins-Domingo *et al.*, 2004; Roger *et al.*, 2011). However, it is not the only culprit and obesity plays an equally strong role when discussing HF etiologies.

1.5.2 Obesity

The American Medical Association's policy recognized obesity as a disease in June 2013 (Ryan & Braverman-Panza, 2014). The prevalence of extreme obesity rose by 2.7% (2000-2010), representing a 70% increase in the United States population. In addition, extreme obesity is 50% higher in women compared to men. Obesity is defined as a chronic, complex and multifactorial condition that arises from a positive energy imbalance over time that is influenced by genetic and environmental factors. Obesity-related inflammation is linked to CVD onset as it is an important contributing factor to heart diseases (Wang & Nakayama, 2010). It is also a key risk factor for the development of hypertension and CAD that both contribute to HF etiology. In fact, obesity may be an independent predictor for HF as it elicits dire effects on cardiac structure and left ventricular systolic pressure while diastolic function is also compromised (Lavie *et al.,* 2009; Lavie *et al.*, 2013). For patients with CHF, obesity is prevalent in 70% of women versus less than half of men (Pyöräiä *et al.*, 2004). Women are also at increased risk of death for even modest weight gain in their childhoods (Hu *et al.*, 2004), while the Framingham Heart Study demonstrated that there was a graded increase in HF risk in both men and women for all body mass index (BMI) categories. A BMI increase for every 1kg/m^2 is correlated with a higher HF risk by 7% in women and 5% in men (Kenchaiah *et al.*, 2002). The duration of obesity is also significant as HF prevalence rates can exceed 70% after 20 years and 90% after 30 years of obesity (Alpert *et al.*, 1997). A graded pattern arises when AHF patients are divided into BMI quartiles: as BMI increases the patients tend to be younger, display more diabetes and hypertension, higher left ventricular ejection fractions; for the fourth quartile (highest BMIs) more tend to be female (Clark *et al.,* 2011; Daniels *et al.*, 2006; Fonarow *et al.,* 2007; Zapatero *et al.*, 2012). HF-related mortality due to obesity likely depends on the presence of left ventricular dysfunction (Gustafsson *et al.*, 2005). The effect of malnutrition on HF is also an important consideration in

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developing countries such as South Africa as malnourished HF patients have an increased in-hospital mortality and high risk of readmission after 30 days (Zapatero *et al.*, 2012).

In summary, AHFS has received limited attention to date and limitations of previous study designs have made it difficult to derive meaningful sex-based comparisons. What is clear is that Africans present with AHF at a younger age during their productive years and that such women suffer at a much younger age than their male counterparts. Acute instances of *de novo* AHF occurring more frequently in women are associated with an increased risk for death and although it does not need multiple co-morbidities to reach this status, females are again at a disadvantage. Women also suffer from diabetes and obesity more than their male counterparts putting them at higher risk for heart insults and the associated consequences. In addition, malnutrition and obesity occur commonly in African women. The rhetorical question therefore remains: why were women so misrepresented in past clinical trials and why is the understanding of AHF pathology so neglected? As the focus of this thesis is also on metabolism the next section of this introduction will briefly highlight normal cardiac metabolism and thereafter AHF pathology will be discussed.

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1.6 Mechanics of the flourishing heart

The amount of adenosine triphosphate (ATP) that the mammalian myocardium can store is low especially when considering that the heart needs to sustain basal metabolism, ionic homeostasis and cardiac contraction in order to pump ~7,000 liters of blood daily (Soukoulis *et al.*, 2009). To meet these demands there is a complete turnover of the myocardial pool ~ every 10 seconds (Neely & Morgan, 1974) and the heart cycles ~6 kg of ATP daily (Neubauer, 2007). This explains why ATP consumed and generated daily is more than fifteen times the heart's own weight (Ingwall, 2002)! Such high demands for energy emphasizes the omnivorous properties of the mammalian heart - it can utilize fatty acids (FAs), glucose, lactate and ketone bodies as energy substrates. The relative contribution to ATP production at any time is tightly controlled and regulated in this exceptionally plastic process that also includes the interdependence of substrates.

For the normal heart that is well perfused and oxygenated (in the absence of pathology) 60-80% of ATP production is contributed by FA β-oxidation (FAO) and the remainder is supplied by carbohydrate (glucose and lactate) catabolism and a small portion from the oxidation of ketone bodies (Bing *et al*., 1954; Neely & Morgan, 1974; Opie, 1968; Opie, 1969). This is all dependent on the availability of oxygen, as glycolysis and glucose oxidation (GO) uses six oxygen molecules to produce 31 ATP molecules, while FAO uses 23 oxygen molecules to produce 105 molecules of ATP. Thus although FAO is an abundant source of energy it is also an inefficient one as more oxygen is used per ATP molecule produced compared to GO. In addition, the FAO cycle produces both $FADH_2$ (1, 5-dihydroflavin adenine dinucleotide) and NADH (nicotinamide adenine nucleotide), whereas GO generates only NADH that is directly linked to ATP production at complex 1 of the electron transport chain (ETC). $FADH_2$ skips complex 1 and so transfers less protons across the ETC making it less efficient in terms of ATP production (Lopaschuk *et al.,* 2010).

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1.6.1 Fatty acid β-oxidation

The free FAs designated for FAO (Figure 1.2) are usually bound to albumin, can be contained in a chylomicron triacylglycerol (TAG) or in a very-low-density lipoprotein (VLDL) in circulation. FAs can cross the cardiomyocyte membrane into the cytosol by simple diffusion or by CD36-mediated transport, fatty acid-transporter protein (FATP) or fatty acid-binding proteins (FABP). After uptake, fatty acyl-CoA synthase (FACS) esterifies FAs to fatty acyl-CoA that potentially has two fates: a) it can be esterified to form TAG complex lipids (bound for storage, an endogenous source of FAs), or b) carnitine palmitoyltransferase- (CPT) 1 can transfer the acyl group to carnitine. Acylcarnitine then enters mitochondria via acylcarnitine translocase (CT) where CPT-2 converts it back to fatty acyl-CoA that can either be cleaved by mitochondrial thioseterase (MTE) to long-chain FA ions (FA^-) that exit via UCP-3 or enters the FAO cycle. Four key enzymes are involved in FAO namely acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-OH-acyl CoA dehydrogenase and 3-keotacyl-CoA thiolase (3-KAT) (Figure 1.3). This cycle produces NADH, $FADH_2$ and acetyl-CoA - the latter entering the tricarboxylic acid (TCA) cycle. The resultant NADH and $FADH_2$ feed into and drive the ETC to produce ATP (discussed later) that is exported to the cytosol via adenine nucleotide translocase (ANT) which forms part of the mitochondrial permeability transition pore (MPTP). It is crucial that FAO be tightly controlled and here malonyl-CoA exerts allosteric inhibition over CPT-1. This is only the downstream result of the “fuel sensor” AMP (adenosine 3', 5'-monophosphate)-activated protein kinase (AMPK), that exerts the main control over cardiac metabolic regulation. When there is an increase/decrease in energy demand, metabolic stress, ATP depletion or a decrease in the creatine/phosphocreatine (Cr/PCr) ratio, AMPK will either stimulate or inhibit acetyl-CoA carboxylase (ACC) to manipulate levels of malonyl-CoA in order to meet metabolic demand (Lopaschuk *et al.*, 2010).

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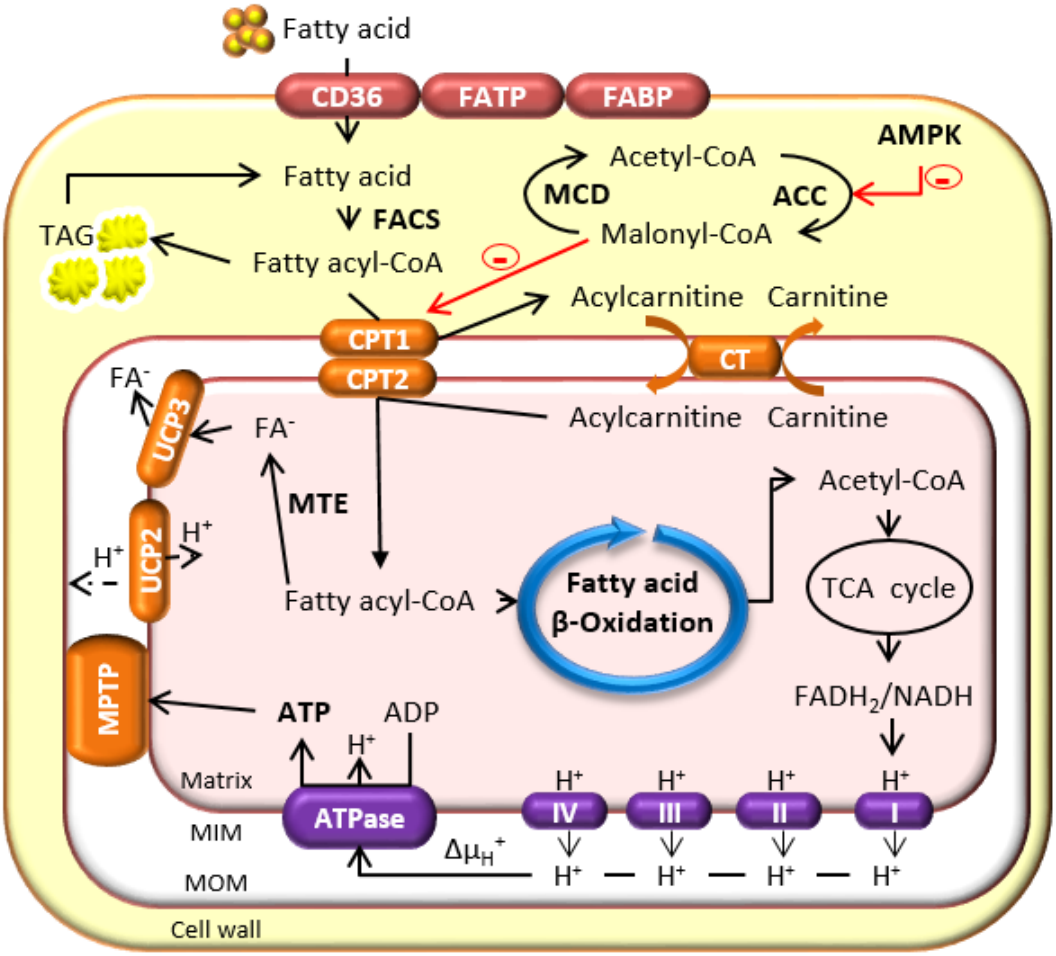

Figure 1.2: Fatty acid β-oxidation in a nut shell. Fatty acids enter cardiomyocytes via diffusion through the CD36/FATP/FABP transporters where in the cytosol fatty acyl-CoA synthase (FACS) converts it to fatty acyl-CoA. This can a) be esterified to triacylglycerol (TAG) or b) carnitine palmitoyltransferase (CPT)-1 transfers an acyl group to carnitine. Carnitinetransferase (CT) shuttles the acylcarnitine into the mitochondrion where CPT-2 transfers it back to fatty acyl-CoA to enter the fatty acid β-oxidation spiral to produce acetyl-CoA to enter the TCA cycle. Resultant $FADH_2$/NADH enters the electron transport chain to produce ATP. The "fuel sensor" AMP-activated protein kinase (AMPK) exerts control over acetyl-CoA carboxylase (ACC) to manipulate levels of malonyl-CoA in order to meet metabolic demands. FATP: fatty acid-transporter protein, FABP: fatty acid-binding protein, MTE: mitochondrial thioesterase, MCD: malonyl-CoA decarboxylase, MPTP: mitochondrial permeability transition pore, UCPs: uncoupling proteins, MIM: mitochondrial inner membrane, MOM: mitochondrial outer membrane, FA^-: fatty acid anions, TCA: tricarboxylic acid, ADP: adenosine 5'-diphosphate ATP: adenosine 5'-triphosphate, $FADH_2$: 1, 5-dihydroflavin adenine dinucleotide, NADH: nicotinamide adenine nucleotide, AMP: adenosine 3', 5'-monophosphate, H^+: proton/hydrogen ion , $\Delta\mu_H^+$: change in electrochemical gradient. Image adapted from Lopaschuk *et al.*, 2010.

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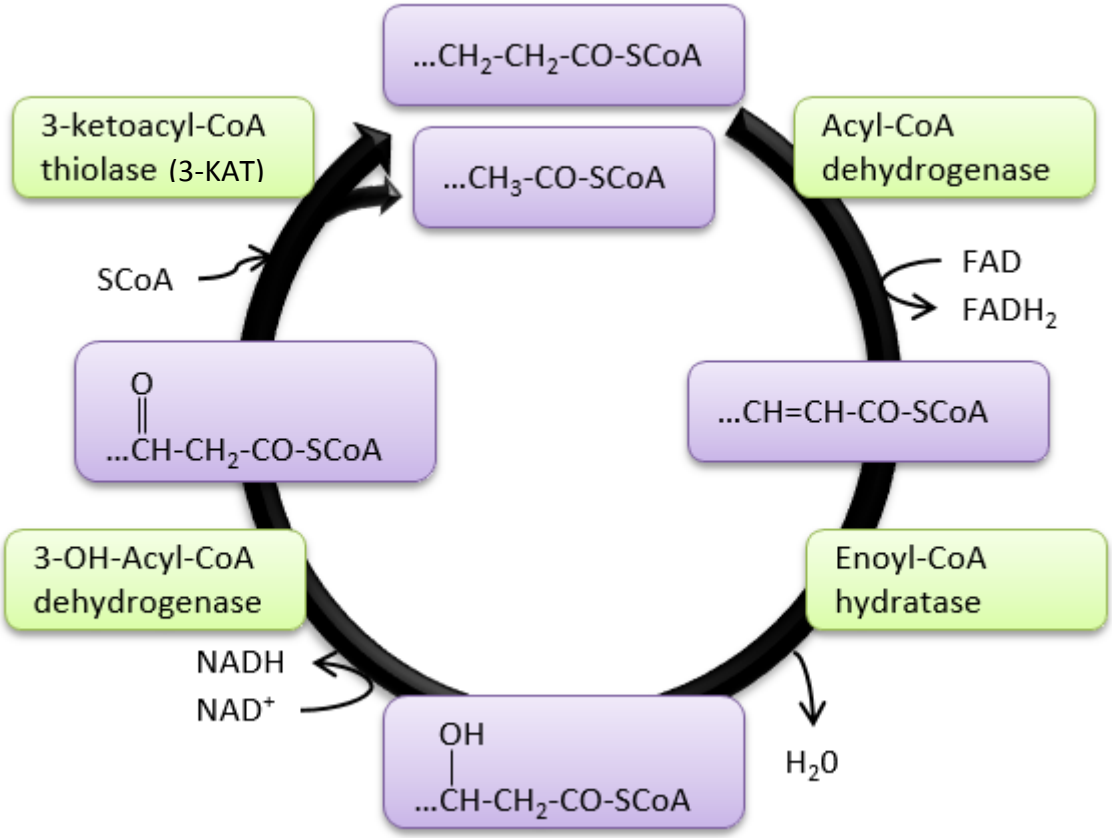

Figure 1.3: Mitochondrial fatty acid β-oxidation spiral. The four enzymes occur with varying fatty acid chain length specificities and have different isoforms. A fatty-acyl chain that is two carbons shorter and acetyl-CoA (destined to enter the TCA cycle) are produced by one cycle of the FA β-oxidation spiral. $FADH_2$: 1, 5-dihydroflavin adenine dinucleotide, NADH: nicotinamide adenine nucleotide, H_2O: water. Image adapted from Lopaschuk *et al.*, 2010.

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1.6.2 Glucose oxidation and the Randle cycle

Glucose is transported into the cytosol after the recruitment of glucose transporter-1 (GLUT-1) and GLUT-4 from the intracellular compartments. AMPK can also intervene in glycolysis as it can trigger GLUT-4 translocation to enhance glucose uptake. Subsequently, glucose is phosphorylated to glucose-6-phosphate (G-6-P) by hexokinase I and/or hexokinase II and phosphofructokinase-1 (PFK-1) catalyzes the reaction to pyruvate. Pyruvate can be anaerobically converted to lactate or it can be transported into mitochondria to undergo oxidation by pyruvate dehydrogenase (PDH) to form NADH (feeds into the ETC) and acetyl-CoA designated for the TCA cycle (Lopaschuk *et al.*, 2010).

Philip Randle and colleagues were the first to describe the reciprocal relationship between FA and carbohydrate metabolism. Since the 1960s, this has been simply referred to as the "Randle cycle" (Figure 1.4) (Garland *et al*., 1963; Randle *et al*., 1963; Randle *et al.*, 1964). This effect lends plasticity to cardiac metabolism for meeting fuel demand and supply. FAO can inhibit GO (pyruvate) in two ways. Firstly, increased acetyl-CoA produced by the FAO cycle can activate pyruvate dehydrogenase kinase (PDHK), that will phosphorylate and inactivate PDH. This leads to backflow in the system resulting in glucose conversion to lactate and associated protons. Secondly, increased acetyl-CoA entering the TCA cycle can also elevate citrate levels that can inhibit cytosolic PFK-1, thereby leading to lowered glucose utilization.

Conversely, GO can also inhibit FAO in two ways: a) 3-KAT (last FAO enzyme) is sensitive to inhibition from increased acetyl-CoA (from GO) and b) elevated acetyl-CoA from GO can be converted to acetylcarnitine by carnitine acetyl transferase (CAT). Cytosolic CAT can convert this into cytosolic acetyl-CoA that is metabolized to malonyl-CoA by ACC, thereby exerting an inhibitory effect on CPT-1 (Lopaschuk *et al.*, 2010).

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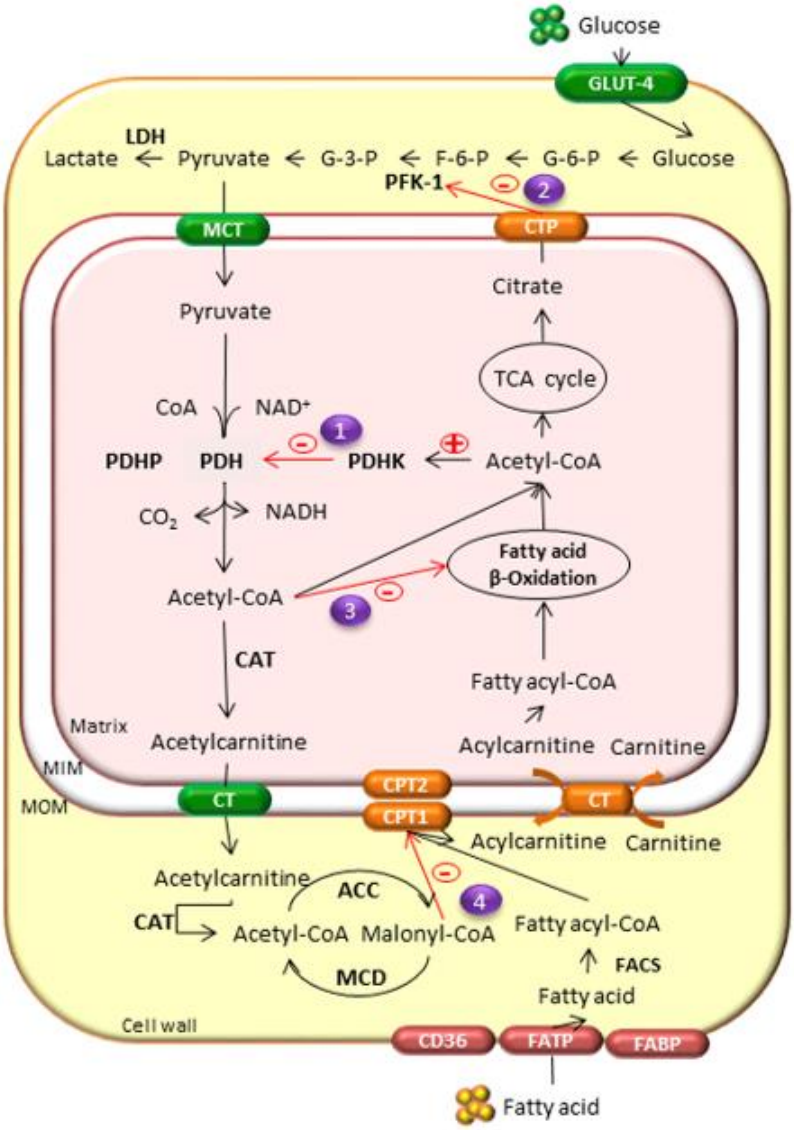

Figure 1.4: The Randle cycle. In order to provide plasticity to cardiac metabolism for exceptional dynamic demands and supplies there is a reciprocal relationship between fatty acid and carbohydrate metabolism. Fatty acid β-oxidation (FAO) can inhibit carbohydrate metabolism in two ways. The increased acetyl-CoA produced by the mitochondrial FAO spiral can either act indirectly on pyruvate dehydrogenase (PDH) (1) or it can translate to citrate produced that feeds back to inhibit phosphofructokinase-1 (PFK-1) (2). Conversely, increased acetyl-CoA produced by glucose metabolism can also inhibit FAO by either targeting the sensitive 3-keotacyl-CoA thiolase (3-KAT) enzyme in the mitochondrial FA β-oxidation spiral (3) or by translating into cytosolic acetylcarnitine that indirectly acts on malonyl-CoA to inhibit carnitine palmitoyltransferase-1 (CPT-1) (4). GLUT-4: glucose transporter-4, G-6-P: glucose-6-phosphate, F-6-P: fructose-6-phosphate, G-3-P: glyceraldehyde-3-phosphate, LDH: lactate dehydrogenase, MCT: monocarboxylate transporter, PDHP: pyruvate dehydrogenase phosphatase, PDHK: pyruvate dehydrogenase kinase, NADH: nicotinamide adenine nucleotide, CO_2: carbon dioxide, CAT: carnitine acetyl transferase, CT: acylcarnitine translocase, ACC: acetyl-CoA carboxylase, MCD: malonyl-CoA decarboxylase, FACS: fatty acyl-CoA synthase, FATP: fatty acid-transporter protein, FABP: fatty acid-binding proteins, TCA: tricarboxylic acid, CTP: citrate transport protein, MIM: mitochondrial inner membrane, MOM: mitochondrial outer membrane. Image adapted from Lopaschuk *et al.*, 2010.

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1.6.3 Electron transport chain

ATP is generated in mitochondria by the high energy reducing equivalents (NADH and $FADH_2$) from the TCA cycle (GO and FAO) that are passed along proteins in the mitochondrial inner membrane (MIM) by a series of redox reactions (Figure 1.5). At complex IV oxygen accepts the hydrogen molecule to form water, but more importantly this reaction produces energy so that other protons are extruded into the intermembrane space from complex I, III and IV. The resultant electrochemical gradient drives complex V (ATP synthase) to phosphorylate adenosine 5'-diphosphate (ADP) to ATP. However, under normal physiological conditions electron flow is not always tightly coupled to ATP generation with some energy being lost as heat. Here uncoupling proteins (UCPs), ANT and non-specific membrane slippage contribute to non-ATPase related proton leaks from the inter-mitochondrial membrane space into the matrix. However, this is a protective feedback mechanism and is sometimes referred to as "uncoupling to survive". Reverse electron transport at complex I (NAD^+ is reduced back to NADH) and electron leaks are natural phenomena occurring within the ETC. Here electrons do not reach the end of the chain to form water, but rather exit early at complex I and III to form superoxide ($SO_2{\cdot}^-$). This natural production of reactive oxygen species (ROS) exacerbates proton leaks and studies found that a decrease in proton gradient due to uncoupling can attenuate $SO_2{\cdot}^-$ production and reverse electron transport (Schwarz *et al.*, 2014). However, when uncoupling becomes dysregulated it may contribute to pathology.

The important role of cardiac metabolism in energy production is abundantly clear and the focus will now shift to AHF pathology with some reference to this context. However, distinct clinical presentations of males and females will initially be discussed, where after the focus will shift to hemodynamic alterations and molecular events.

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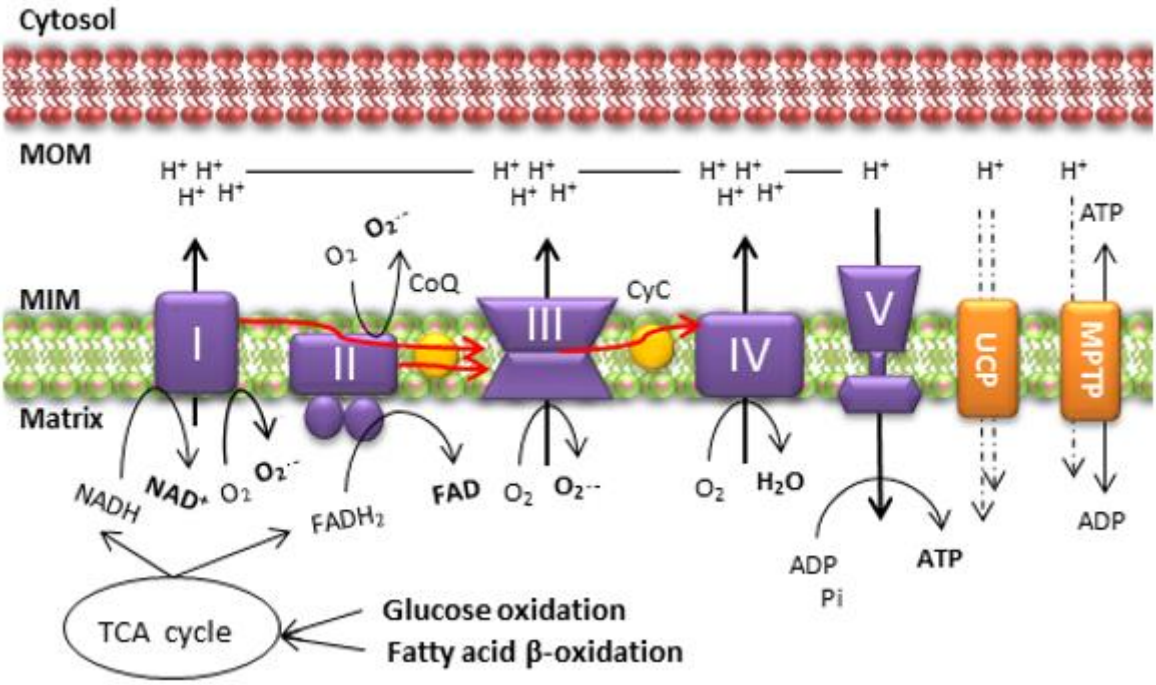

Figure 1.5: Mitochondrial respiratory/electron transfer chain. Reducing equivalents NADH and $FADH_2$ from GO and FAO are passed to and through (via coenzyme Q and cytochrome c shuttles) the electron transport chain as high energy loaded electrons and these electrons release energy as it undergoes redox reactions at the copper and iron centers of the individual complexes in the chain. Hydrogen ions are extruded into the inter-membrane space from the matrix due to related conformational changes. The availability of oxygen is a rate-limiting step, as it is the terminal acceptor of energy depleted electrons. The electron gradient drives ATP synthase to phosphorylate ADP to ATP. $FADH_2$: 1, 5-dihydroflavin adenine dinucleotide, NADH: nicotinamide adenine nucleotide, H_2O: water, O_2: oxygen, ADP: adenosine 5'-diphosphate , ATP: adenosine 5'-triphosphate, UCPs: uncoupling proteins, MPTP: mitochondrial permeability transition pore, TCA: tricarboxylic acid, I-V:the five protein complexes of the electron transport chain, CoQ: coenzyme Q/ubichinone, CyC: cytochrome c, H^+: proton/hydrogen ion, MIM: mitochondrial inner membrane, MOM: mitochondrial outer membrane. Image adapted from Schwartz *et al.*, 2014.

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1.7 Pathology of acute heart failure

AHF pathology can be sub-divided into two categories (Table 1.3). Here acute decompensated (cardiac) failure is characterized by a milder and more gradual nature whereby symptom worsening presents. The presence of pulmonary edema is infrequent, while symptoms such as jugular vein congestion, peripheral edema, poor peripheral perfusion and renal and hepatic dysfunction are frequent. Such patients are usually younger males with normal/low range blood pressure with on-going myocardial injury (ischemia) as a precipitating factor. By contrast, acute cardiovascular (hypertensive) failure is a rapidly progressive disorder that is characterized by extreme and acute pulmonary edema and dyspnea. Such patients are usually women that present with high blood pressure, preserved ejection fraction and hypertension - this form is common for *de novo* AHF (Cotter, *et al*., 2008; Phan *et al*., 2012).

	Acute decompensated (cardiac) failure	**Acute cardiovascular (hypertensive) failure**
Sex	Usually younger males	Usually women Common for *de novo* AHF
Progression	Milder and more gradual symptom worsening presents (days)	Extreme and acute: a rapidly progressive disorder (hours)
Precipitating factor	On-going myocardial injury (ischemia)	Hypertension
Main mechanism of onset	↓ Contractility and renal hypoperfusion	↑ Afterload
Main symptom	Fatigue	Dyspnea
Main sign(s)	Peripheral edema, jugular venous stasis, hepatomegaly	Extreme and acute pulmonary edema, dyspnea, Rales
Systolic blood pressure	Normal or low	Normal or high
Left ventricular ejection fraction	Low	Normal
Left ventricular filling pressure	May be reduced by low cardiac output	High
Cardiac output	Low	Normal or high

Table 1.3: Classifications of acute heart failure (AHF). Males and females in the clinical setting present with two different classifications of AHF. Adapted from Metra *et al*., 2010.

This review will focus on the female AHF phenotype as it is the main interest of this thesis. The proposed pathology encompasses the most important symptoms whereby AHF females present,

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namely fluid overload and dyspnea, left ventricular (diastolic) failure, left ventricular preserved ejection fraction and high systolic blood pressure. Thereafter co-morbidities (obesity and diabetes) will be discussed - these parameters will be separately described but as such components are intertwined none of them can easily be considered in isolation.

1.7.1 Hemodynamics

Poor contractility is often present during AHF together with a resultant low cardiac output and decreased plasma volume. Pathways such as the renin-angiotensin-aldosterone-system (RAAS) and adrenergic and inflammatory pathways are activated to try and increase the plasma volume. However, fluid retention can be exacerbated by pharmacological treatment, non-compliance and diet. Although mechanisms such as B-type natriuretic peptide (BNP) try to restore euvolemia, fluid retention and accumulation persists (Pandit *et al.,* 2011). As mentioned previously, AHF patients are mainly hospitalized for dyspnea and not for low cardiac output. Pulmonary edema and congestion are the main mechanisms causing dyspnea (Metra *et al.*, 2010).

Pulmonary edema arises when the colloid osmotic pressure (provided by plasma proteins) is exceeded by intravascular pressure (hydrostatic force) (Figure 1.6). Fluid from the intravascular space is then forced into the alveoli and this increases resistance in the airways and renders the lung incompliant, so leading to difficulty with breathing and increased cardiac effort (Haydock & Cowie, 2010). Pulmonary congestion is pulmonary venous hypertension or increased pulmonary capillary wedge pressure that results in pulmonary interstitial and alveolar edema (Gheorghiade & Pang, 2009).

A potential mechanism whereby this may occur is that fluid may be redistributed from the periphery to the pulmonary system. However, this is not the only cause as pulmonary congestion/edema and increased pulmonary capillary pressure can also arise due to vasoconstriction, particularly when diastolic dysfunction is present. Venoconstriction results in greater right heart venous return and in combination with failing ventricles causes increased end diastolic pressures in both right and left

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ventricles. Arterial vasoconstriction results in increased afterload in the left ventricle with a subsequent decrease in stroke volume. The exact trigger for such excessive vasoconstriction has not been fully clarified, however, inflammation and neurohormonal activation are favored as potential mediators (Colombo *et al*., 2008; Metra *et al.*, 2010). Pulmonary congestion may also be caused by an acute increase in blood pressure (afterload) in the presence of diastolic dysfunction (Cotter *et al.*, 2002; Cotter *et al.,* 2008) and by an increase in left ventricular diastolic pressure. It can also occur indirectly via activation of neurohormones and by remodeling (Gheorghiade *et al.*, 2006).

Pulmonary capillary wedge pressure (high left side pressures) can cause increased systemic venous pressure (at the high right atria) that can also contribute to cardio-renal syndrome (Damman *et al.*, 2007; Núñez *et al.,* 2015). West and colleagues (2010) postulate that dyspnea more likely results from incompatible information within the controls of the respiratory system. They propose that patients perceive a mismatch between the afferent sensation (mechanical receptors in the chest wall, lungs and airways, and chemoreceptors in the blood) and efferent motor activity (in the brain that descends down into the diaphragm and supporting respiratory muscles) (West *et al*., 2010). In summary, dyspnea (a key characteristic of AHF) arises due to rapid dysregulation of cardiac pressures and associated compensatory mechanisms that may arise.

Venous congestion is starting to gain credibility as a primary contributor to ADHF, rather than being considered a secondary consequence. Fluid redistribution and accumulation are two mechanisms proposed for venous congestion pathogenesis. Neurohormonal [increased levels of endothelin-1 (ET-1), angiotensin II (AII) and norepinephrine] and endothelial activation [increased levels of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-alpha (TNF-α)] in AHF increases oxidative stress (OS), inflammation and vasoconstriction (Colombo *et al.*, 2015).

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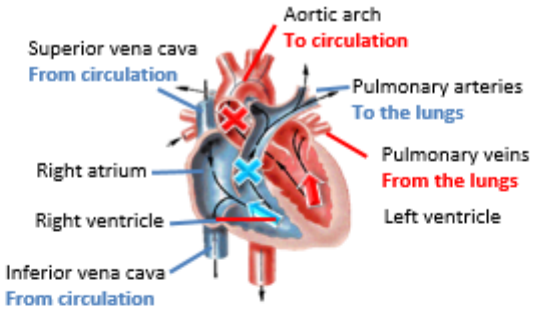

	Right HF	Left HF
Reduced ventricular contractility causes backup/backflow to:	Extremities	Lungs
Decreased blood flow to:	Lungs	Circulation
Resulting symptom	DNV, edema	Dyspnea, crackles

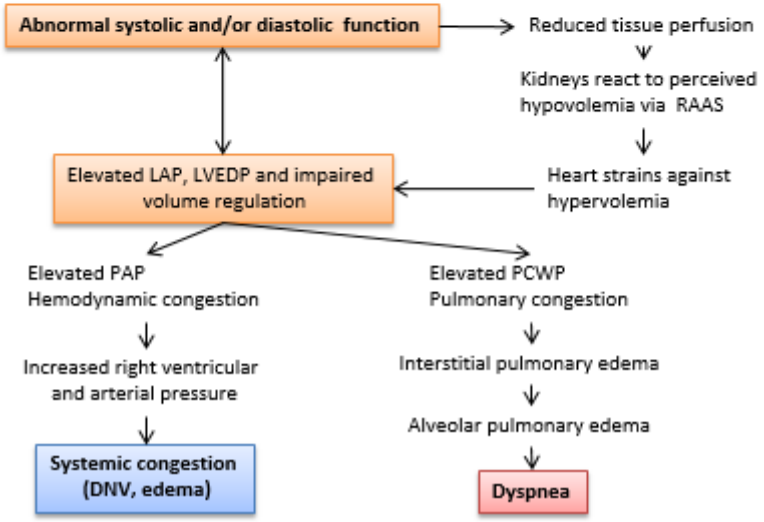

Figure 1.6: Hemodynamic pathophysiology of systemic and pulmonary edema. HF: heart failure, DNV: distended neck veins, RAAS: renin-angiotensin-aldosterone-system, LAP: left atrial pressure, LVEDP: left ventricular end diastolic pressure, PAP: pulmonary arterial pressure, PCWP, pulmonary capillary wedge pressure. Image adapted from Hummel *et al.*, 2015.

Endothelial cells are activated by biomechanical forces due to wall stress (circumferential or shear) and/or hydrostatic pressure. These cells then phenotypically switch from a quiescent to a vasoconstricted, pro-inflammatory, pro-oxidant activated state. Biomechanical stress to endothelial cells causes increased ROS production and associated endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) up regulation and activity. Increased ROS can either interact with the elevated nitric oxide (NO) levels to produce reactive nitrogen species (RNS), or ROS can

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oxidatively degrade NO thereby leading to vasoconstriction. It is also possible that ROS and cytokines can trigger the nuclear factor (NF)-κB inflammatory response that would lead to cyclo-oxygenase-2 (COX-2) expression. This led Colombo *et al.* (2015) to put forward that the above-mentioned fluid redistribution (vascular pathway) and fluid accumulation pathway do not act in isolation, but that rather that there is a positive feedback system cycling between the two pathways. This results in a mismatch between pre- and afterload and cardiac function that leads to ADHF (Colombo *et al.*, 2015).

Fluid redistribution and fluid accumulation are also linked to increased arginine vasopressin (AVP) release. Abnormal release of vasopressin overrides AVPs osmotic regulation and leads to additional water absorption so contributing to hyponatremia in AHF (Cotter *et al.*, 2008; Pearse & Cowie, 2014; Verbrugge *et al.*, 2015). AHF volume and pressure overloads (but not normal hearts) show increased TNF-α levels and this has received some attention - it seems TNF-α exerts its effect by inducing NO and/or by dampening the Ca^{2+} concentration during systole. TNF-α in HF-related studies has been associated with premature death, effects on β-adrenergic receptors and left ventricular remodeling and contractile dysfunction (Chen *et al*., 2008).

Left ventricular diastolic dysfunction is regularly associated with a combination of two mechanisms (Laribi *et al.*, 2012). Firstly, reduced left ventricular dispensability occurs together with the associated pulmonary diastolic blood pressure (PDBP) that is upstream of increased left ventricular end diastolic pressure. These are, however, a consequence of intrinsic muscle impairment (i.e. ventricle rigidity due to diabetes) and/or left ventricular remodeling that frequently results in pressure overload (systolic blood pressure). For the second mechanism there is altered ventricular filling due to relaxation time elongation in situations of hypertension and concentric left ventricular remodeling. This would occur during fluid overload or arrhythmias in which case the filling time is decreased and the resultant (inadequate) filling leads to increased pressure upstream and finally to a reduced ejection fraction (Laribi *et al.*, 2012).

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Left ventricular failure is a vicious cycle where initial compensatory mechanisms aimed at temporary relief exacerbates pathology in the long run. With left ventricular failure, decreased renal perfusion activates RAAS that initially assists in maintaining cardiac output but is detrimental in the long run. RAAS is responsible for increased vasoconstriction (initially maintains vital organ perfusion), sodium (Na^+) and water reabsorption and retention to enhance ventricular preload and also for interstitial fibrosis. These mechanisms all have the important outcomes of causing vasoconstriction, eccentric ventricular hypertrophy, Na^+ retention and myocardial fibrosis. Aldosterone is also responsible for eccentric ventricular hypertrophy, Na^+ retention and fibrosis in addition to coronary vasculopathy and cytokine activation. Left ventricular failure also triggers arterial baroreceptors to detect the reduced cardiac output and so initiates increased sympathetic activation by increasing norepinephrine levels. This sympathetic intervention leads to enhanced contractility, heart rate and wall stress. Myocardial hypertrophy occurs as a compensatory mechanism with the aim to preserve the wall stress as the ventricle dilates. However, this causes ventricular remodeling that feeds back to the start of the chain reaction to exacerbate the initial left ventricular failure. Ventricular dilation and volume expansion occurs in an attempt to increase ventricular preload, a compensatory mechanism based on the Frank-Starling mechanism. Increased diastolic filling and stretch results in a more forceful contraction therefore increased stroke volume and cardiac output (Haydock & Cowie, 2010; Krum & Abraham, 2009).

Left ventricular and arterial stiffness is common in patients with preserved ejection fraction. Such individuals are extremely sensitive to even a remote rise in volume (preload and afterload) as they generally exhibit high diastolic pressures in small diastolic volumes. Thus a slight increase in volume could have one of two consequences or a combination thereof: a) a robust increase in left ventricular end diastolic pressure and b) an elevation in relative afterload causes afterload mismatch that significantly slows left ventricular active relaxation. This “mismatch” refers to a discrepancy between the increasing afterload and impaired systolic performance. This results in increased left ventricular end diastolic pressure and reduced cardiac output. Slowing of relaxation leads to the

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cardiac pressures transferring back to the pulmonary system; the pulmonary venous pressure in turn is transferred back to the alveoli and in this way contributes to pulmonary edema (Cotter *et al.*, 2008; Phan *et al.*, 2012).

The elevated systolic blood pressure occurring in AHF female patients can have one of two roles in pathology. Here it can exacerbate an already dire situation by undue vasoconstriction and increased afterload. But as this is more common in women with preserved left ventricular ejection fraction, it may well be a by-product of the pre-existing AHF via neurohormonal and cardiac activity (Metra *et al.*, 2010).

In summary, dyspnea or pulmonary congestion arises in response to erratic cardiac pressures and the resulting activation of compensatory mechanisms, whereas systemic or venous congestion is caused by a mismatch between pre- and afterload. Left ventricular failure is the pathological outcome of prolonged compensatory mechanisms and left ventricular diastolic dysfunction is the combined result of left ventricular reduced dispensability and altered ventricular filling. Lastly, patients with preserved ejection fraction are exceptionally sensitive to small increases in volume as this results in pre- and afterload mismatch thereby fueling the vicious cycle. Co-morbidities (obesity and diabetes) will now be discussed – bear in mind that these should ideally not be considered in isolation even though this review attempts to describe them separately.

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1.7.2 Molecular effects of co-morbidities

1.7.2.1 Obesity

Being saddled with obesity can result in far-reaching and varied effects, including cardiac structure and function, neurohormonal and cellular effects and both central and peripheral hemodynamics (Lavie *et al.*, 2013). Excess body weight correlates proportionately and positively with total blood volume and cardiac output (Alexander & Alpert, 1998; Alpert, 2001). Increased left ventricular stroke volume and work is responsible for the higher cardiac output and work (Alpert, 2001). Left ventricular failure and associated diastolic dysfunction is common in obese patients (Alexander, 1964; Lavie *et al.*, 2009), while in the presence of hypertension it was found that concentric hypertrophy predominates (Alpert *et al*., 1998; Messerli *et al.*, 1983a; Messerli *et al.*, 1983b). Lavie *et al.*, 2013 proposed that excessive adipose accumulation leads to increased fat-free mass and decreased systemic vascular resistance, followed by higher circulating blood volume, left ventricular stroke volume and cardiac output. In parallel, there is no perceivable change in heart rate. Left ventricular enlargement, together with hypertension result in increased wall stress causing left ventricular hypertrophy. Mild/inadequate hypertrophy leads to left ventricular systolic dysfunction while severe/adequate hypertrophy results in left ventricular diastolic dysfunction. The excessive adipose accumulation observed could also result in sleep apnea, while obesity hypoventilation syndrome leads to hypoxia and/or acidosis that culminates in pulmonary arterial hypertension (and increased cardiac output) that causes right ventricular hypertrophy and enlargement. Left ventricular failure can also operate on a feedback basis to cause pulmonary venous hypertension leading to pulmonary arterial hypertension resulting in right ventricular failure. Neurohormonal changes associated with obesity are insulin resistance and hyperinsulinemia, leptin insensitivity and hyperleptinemia, reduced adiponectin, sympathetic nervous system activation, activation of RAAS and overexpression of peroxisome proliferator-activator receptor (PPAR) (Lavie *et al.*, 2013).

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Adipocyte endocrinology as a new research field only really gained momentum after 1994 when Zhang and colleagues found that adipocytes produced the hormone leptin (Zhang *et al.*, 1994). Thus it is now clear that adipose tissue is not a mere energy reservoir but an active endocrine organ with far-reaching effects. Obesity can therefore now be linked to CVD via its inflammatory role with inflammatory cytokines (produced by adipocytes and macrophages) secreted when macrophages are activated due to abnormal levels of lipids, FAs and cytokines. Leptin, adiponectin, TNF-α, IL-1 and IL-6 make up the inflammation-related adipokines, while pro-coagulant substances include plasminogen activator inhibitor−1 (PAI-1). Vasoactive substances include leptin, angiotensinogen and endothelin. Various modulators that may contribute to insulin resistance are also secreted by adipocytes, e.g. FAs, TNF-α and resistin (Wang & Nakayama, 2010). In obese individuals macrophage accumulation (Weisberg *et al.*, 2003) and activation result in inflammatory cytokine secretion (Fried *et al.,*1998) that reaches the liver via the portal circulation and leading to hepatic inflammation (Tarakçioglu *et al*., 2003). This in turn can induce a systemic inflammatory response that together with the effects of hypertension, diabetes and hypercholesterolemia can trigger endothelial dysfunction. Subsequently, there is the release of a variety of mediators and surface proteins, vascular tone changes and increased fibrinolysis and coagulation. With the additive effects of obesity-induced systemic OS this ultimately results in atherosclerosis - a key risk factor for developing CVD (Wang & Nakayama, 2010).

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1.7.2.2 Diabetes

In situations of hemodynamic disruption e.g. AHF there will be serious consequences as kidney function is highly dependent on the hemodynamics of a patient. Venous pressure, hypotension, neurohormonal activation and renal hypoperfusion are all part of an interconnected web whereby AHF causes kidney dysfunction (Metra *et al.*, 2010). This can occur in the absence of CAD and here the left ventricle displays hypertrophy together with systolic and diastolic dysfunction - the so-called diabetic cardiomyopathy (DCM) (Poornima *et al.,* 2006).

DCM is a clinical condition where cardiovascular damage presents in diabetic patients. It is characterized by left ventricular hypertrophy, systolic and diastolic dysfunction and myocardial dilation (Voulgari *et al*., 2010). DCM is independent of the presence of ischemic heart disease and hypertension, although hypertension frequently co-exists and accelerates the underlying functional pathology. DCM is an exceptional condition with far-reaching influences and intertwined pathology, of which the full discussion lies outside the scope of this review (Poornima *et al.*, 2006; Voulgari *et al.*, 2010). The three hallmarks of DCM, namely obesity, insulin resistance and hyperglycemia will be briefly covered in the following paragraphs.

Obesity (increased FAs and triglycerides [TG]) is a predisposition for type 2 diabetes, dyslipidemia and hypertension. Higher FA production by the adipose tissue due to increased leptin, sympathetic innervation and TNF-α is implicated in lipotoxicity, apoptosis, insulin resistance and myocardial contractile dysfunction. Increased FAs can lead to insulin resistance by activation of atypical protein kinase C (PKC) that impairs insulin transduction through the insulin receptor substrate-1 (IRS-1) and the phosphatidylinositol 3-kinase (PI3-K) pathway (Kim *et al.*, 2001). FAs can also prevent the activation of protein kinase B (AKT-1) through up regulation of the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Schwartzbauer & Robbins, 2001). The reaction between the palmitoyl-CoA (an intracellular FA intermediate) and ceramide can result in lipotoxicity (lipid-induced apoptosis) (Unger & Orci, 2000; Zhang *et al.*, 2001). Myocardial contractility can also be directly

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altered by FAs though ATP-sensitive potassium channel (K_{ATP}) channel opening (Unger & Orci, 2000). Obesity is also marked by reduced levels of adiponectin (insulin-sensitizing modulator) and may therefore contribute to insulin resistance onset (Yamauchi *et al.*, 2001). Insulin resistance is associated with elevated levels of C-reactive protein (CRP) (a marker of the acute phase of inflammation) and this is associated with increased DCM risk (Festa *et al.*, 2000; Ridker *et al*., 2000). The catastrophic relationship between over nutrition (obesity) and cardiac insulin resistance can loosely be divided into four cellular events (Figure 1.7). It is noteworthy that the described pathological situations all revolve around the phosphorylation of serine residues of IRS-1. This decreases phosphorylation of tyrosine on IRS-1, association with PI3-K (p85 subunit) and it triggers its proteasome–dependent degradation (Aroor *et al*., 2012).

Insulin resistance results in compensatory hyperinsulinemia, another hallmark of diabetes that can trigger multiple pathways that can lead to cardiac hypertrophy. The PI3-K/AKT-1 pathway stimulates one of two paths in order to increase the nuclear transcription of the hypertrophy program, i.e. it can deactivate glycogen synthases kinase-3β (GSK-3β) (Morisco *et al.*, 2005) or activate the mammalian target of rapamycin (mTOR) (Khamzina *et al*., 2005). Hyperinsulinemia may also enhance AKT-1 activation through increased stimulation of the sympathetic nervous system via protein kinase A (PKA) and calcium-calmodulin dependent kinase (CaMK) (Morisco *et al.*, 2005). In order to increase the nuclear transcription of the hypertrophic response, Rho and Ras can be prenylated (directly or indirectly) via the p38 mitogen-activated protein kinase (MAPK) pathway (Wang *et al.,* 2004). Of note, it is simplistic to only implicate hyperinsulinemia in the onset of cardiac hypertrophy. Insulin resistance disrupts multiple systems that interact to cause, among others, myocardial cell death, contractile dysfunction, attenuated ATP production and decreased cardiac efficiency. Eventually the heart succumbs to the demands placed on it and fails (Figure 1.8) (Aroor *et al.*, 2012).

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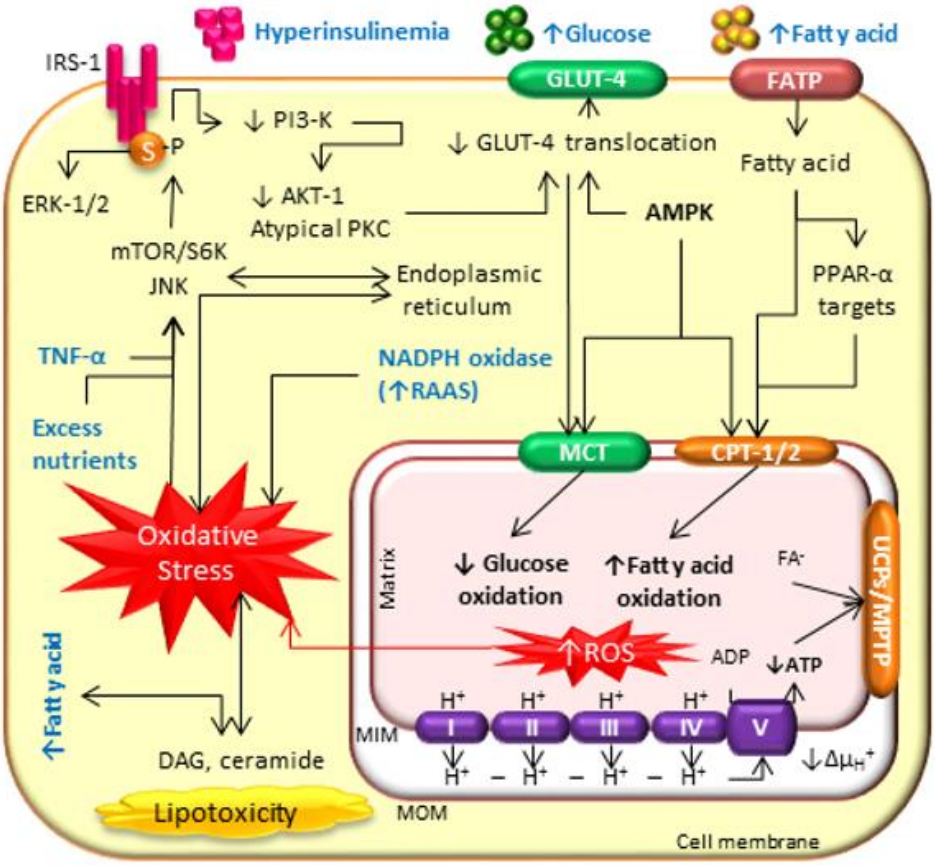

Figure 1.7: Cellular events as obesity (over nutrition) contributes to impaired insulin signaling. (1) Over nutrition results in increased levels and uptake of fatty acids, diacylglycerol (DAG) and ceramide accumulates that phosphorylates serine of insulin receptor substrate-1 (IRS-1). Hyperglycemia can also trigger the phosphorylation of IRS-1, activate protein kinase C (PKC) and upregulate angiotensin II. Excess glucose and amino acids both activate the mammalian target of rapamycin (mTOR)/S6 kinase (S6K). (2) Dysregulation of adipocyte function increases secretion of inflammatory markers such as interleukin-6 and tumor necrosis factor-alpha (TNF-α) that activate mitogen-activated protein kinase, PKC, mTOR/S6K and IRS-1 degradation mediated by suppressor of cytokine signaling-3. (3) Through renin-angiotensin-aldosterone-system (RAAS) activation, aldosterone and AngII activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complexes to generate radical oxygen species (ROS) that stimulate isoenzymes, MAPK and S6K, all able to phosphorylate IRS-1. It is also suggested that cross talk exists between RAAS and mTOR/S6K. (4) Mitochondrial stress acts through serine phosphorylation of IRS-1. ER stress is proposed to contribute via MAPK C-Jun N-terminal kinase (JNK) activation. ERK-1/2: extracellular signal-regulated kinase-1/2, PI3-K: phosphatidylinositol 3-kinase, AKT-1: protein kinase B, PKC: protein kinase C, GLUT-4: glucose transporter-4, AMPK: adenosine 3', 5'-monophosphate-activated protein kinase, FATP: fatty acid-transporter protein, PPAR-α: peroxisome proliferator activated receptor- alpha, MCT: monocarboxylate transporter, CPT-1/2: palmitoyltransferase-1/2, FA^-: fatty acid anions, ADP: adenosine 5'-diphosphate, ATP: adenosine 5'-triphosphate, UCPs: uncoupling proteins, MPTP: mitochondrial permeability transition pore, H^+: proton/ hydrogen ion, $\Delta\mu_H^+$: change in electrochemical gradient, MIM: mitochondrial inner membrane, MOM: mitochondrial outer membrane. Image adapted from Aroor *et al.,* 2012.

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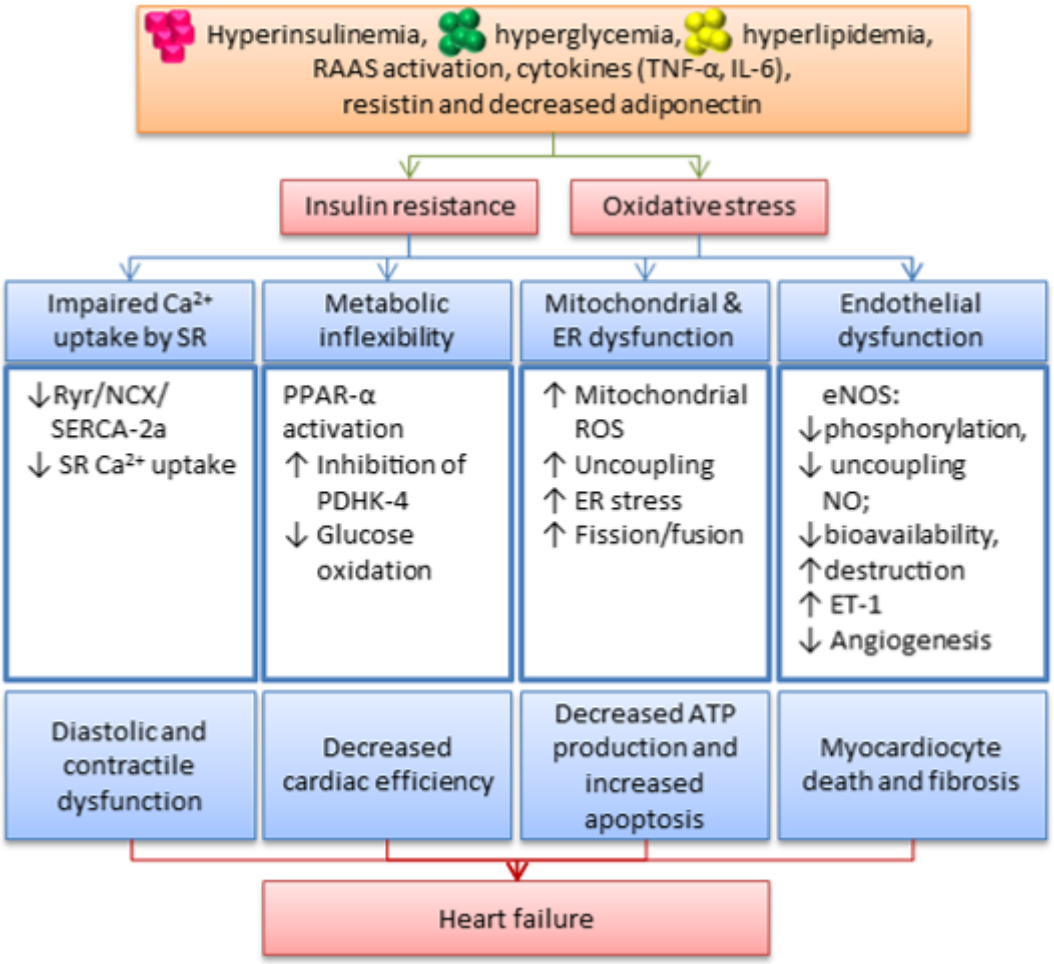

Figure 1.8: Dysfunctional roads to heart failure due to insulin resistance and associated oxidative stress. Insulin resistance and all its cofactors converge to cause heart failure by mainly targeting calcium handling in the sarcoplasmic reticulum (SR), flexibility of cardiac metabolism, mitochondrial and endothelial function. RAAS: renin-angiotensin-aldosterone-system, TNF-α: tumor necrosis factor-alpha, IL-6: interleukin-6, Ca^{2+}: calcium, ER: endoplasmic reticulum, RyR: ryanodine receptors, NCX: Na^{+}/Ca^{2+} exchanger, SERCA-2a: sarco(endo)plasmic reticulum calcium-ATPase-2a, PPAR-α: peroxisome proliferator activated receptor- alpha, PDHK-4: pyruvate dehydrogenase kinase-4, ROS: radical oxygen species, eNOS: endothelial nitric oxide synthase, NO: nitric oxide, ET-1: endothelin-1, ATP: adenosine 5'-triphosphate. Image adapted from Aroor *et al.*, 2012.

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Hyperglycemia leads to increased GO and production of mitochondrial $SO_2^{\cdot -}$ in mitochondria (Nishikawa *et al.*, 2000). Hyperglycemic-induced injury can be mediated by alternative pathways that are activated when glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is inhibited by poly (ADP-ribose) polymerase (PARP) in response to $SO_2^{\cdot -}$ -mediated deoxyribonucleic acid (DNA) damage. Glucose is subsequently diverted away from glycolysis into non-oxidative glucose pathways (NOGPs) i.e. advanced glycation end products (AGEs), hexosamine biosynthetic pathway (HBP), polyol and PKC pathway. This can lead to hypertrophy, increased ventricular stiffness, impaired filling and relaxation and prolonged Ca^{2+} transients by mechanisms involving collagen crosslinking, altered function and expression of ryanodine receptors (RyR) and sarco(endo)plasmic reticulum calcium-ATPase-2 (SERCA-2) and increases in extracellular matrix (Bidasee *et al.*, 2003; Clark *et al.*, 2003; Herrmann *et al*., 2003; Wakasaki *et al.*, 1997). The effects of AGEs can be direct and also be mediated through specific receptors (RAGE) to activate TNF-α, VCAM-1, NF-κB, and interleukin that contribute to the inflammatory component of pathology (Ban & Twigg, 2008). Thus hyperglycemia activates multiple pathophysiological mechanisms (Mapanga & Essop, 2015) that eventually contributes to the characteristic pathology found with DCM (Dei Cas *et al.*, 2015) (Figure 1.9).

Although DCM occurs independently of hypertension, these commonly co-exist in a synergistic manner and as a result there are shared pathologic pathways. For example, dyslipidemia (characteristic of DCM) also plays a key role in hypertension. Low-density lipoprotein (LDL) ultimately exacerbates and promotes vasoconstriction as the oxidized form suppresses endothelial NO production, thereby stimulating constriction. In addition, vasorelaxation is impaired by hypertriglyceridemia (Goode *et al*., 1995).

This overview briefly provided a synopsis of the key hallmarks of DCM, namely obesity, insulin resistance and hyperglycemia. The central theme arising from this discussion is the significant, causative role played by altered metabolism. The next (short) section will summarize clinical data

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regarding the contributions of altered cardiac energy status due to conditions such as obesity, diabetes and sex.

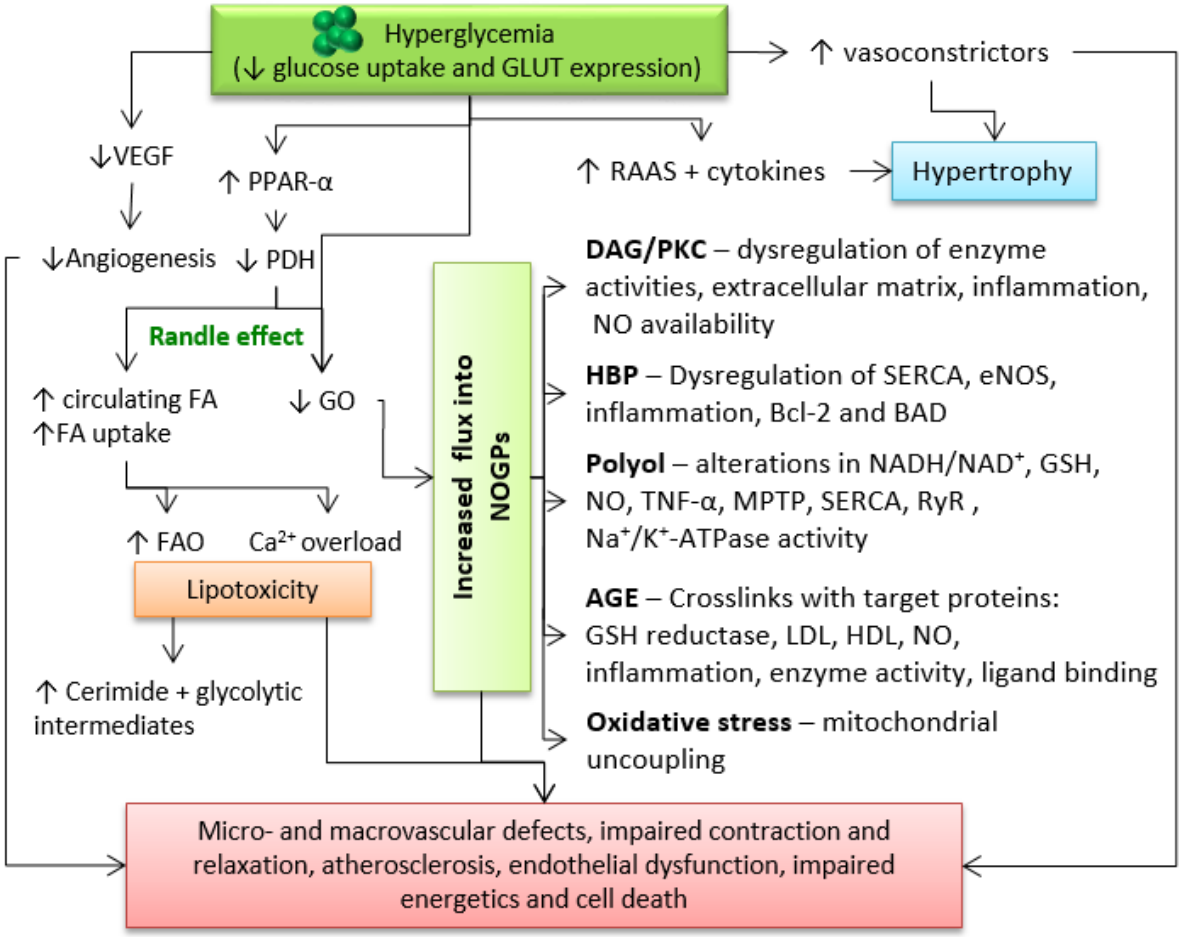

Figure 1.9: Characteristic pathological pathways activated by hyperglycemia in diabetic cardiomyopathy (DCM). Hyperglycemia has direct and indirect effects on the myocardium. Most notably is the decreased glucose oxidation (GO) and increased glucose flux into non-oxidative glucose pathways (NOGPs) that result in multiple pathologies. GLUT: glucose transporter, VEGF: vascular endothelial growth factor, PPAR-α: peroxisome proliferator activated receptor- alpha, PDH: pyruvate dehydrogenase, FA: fatty acid, FAO: fatty acid oxidation, Ca^{2+}: calcium, RAAS: renin-angiotensin-aldosterone-system, DAG/PKC: diacylglycerol/protein kinase C, NO: nitric oxide, HBP: hexosamine biosynthetic pathway, SERCA: sarco(endo)plasmic reticulum calcium-ATPase, eNOS: endothelial nitric oxide synthase, Bcl-2: B-cell lymphoma-2, BAD: Bcl-2-associated death promoter, $NADH^{+}$: nicotinamide adenine nucleotide, GSH: glutathione, TNF-α: tumor necrosis factor-alpha, MPTP: mitochondrial permeability transition pore, RyR: ryanodine receptors, Na^{+}/K^{+}: sodium/potassium, AGE: advanced glycation end products, LDL: low-density lipoprotein, HDL: high-density lipoprotein. Image adapted from Dei Cas *et al.*, 2015.

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1.7.3 Metabolism of the failing heart

Females are known to have enhanced cardiac work, lower efficiency, greater myocardial blood flow and higher oxygen consumption (Chareonthaitawee *et al.*, 2001; Herrero *et al.*, 2005). In parallel, they display higher myocardial FA utilization and oxidation but lower glucose utilization (Desrois *et al.*, 2004; Peterson *et al.*, 2007; Peterson *et al.*, 2008). Here the greater percentage body fat ensures increased FA release into circulation and therefore greater myocardial FA delivery likely contributes to such metabolic differences (Mittendorfer, 2005). Substrate competition (FAs vs. glucose) may explain the lower glucose utilization in females although the role of sex hormones should not be overlooked. Here studies demonstrated that estrogen decreases GO, gluconeogenesis and glycogenolysis, but increases FAO in skeletal muscle and the liver (Kendrick & Ellis, 1991; Mittendorfer, 2005). Estrogen may also increase cardiac oxygen consumption by uncoupling mechanisms (Peterson *et al.*, 2007; Peterson *et al.*, 2008). Whatever the explanation, such metabolic differences become even more apparent in pathological conditions (HF, hypertrophy, diabetes and obesity) that affect cardiac metabolism.

Diabetes and obesity are conditions where insulin resistance occurs and both involve alterations to myocardial FA and glucose metabolism, cardiac steatosis and phosphate energy metabolism (Table 1.4) (Iozzo, 2010). Moreover, FA metabolism is positively correlated to BMI and insulin resistance for females (but not males). Glucose availability is lowered by the interaction of myocardial blood flow and oxygen consumption – obesity and female gender are independent predictors for both (Knuuti *et al.*, 2001; Peterson *et al.*, 2004; Peterson *et al.*, 2008). With especially type 2 diabetes and obesity, peripheral insulin resistance increases plasma FA levels thereby leading to higher FA delivery to the myocardium and thus increased FA uptake and oxidation. GLUT expression and insulin-mediated glucose transport are both decreased under such circumstances (Boudina & Abel, 2007; Young *et al.*, 2002). Type 2 diabetes and obesity are also characterized by cardiac stenosis that further exacerbates the metabolic shift by providing additional FAs (Kankaanpää *et al.*, 2006; McGavock *et*

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al., 2007; Rijzewijk *et al.*, 2008; Szczepaniak *et al.*, 2003). Moreover, insulin resistance and obesity result in significant declines of the phosphocreatine/ATP (PCr/ATP) ratio (Perseghin *et al.*, 2007).

Myocardial substrate metabolism in clinical studies							
	Fasting GU	Insulin GU	FAU & oxidation.	TG depot	Oxygen use	Efficiency	PCr/ATP
Female gender	↓	→	↑	↓	↑	↓	→
Obesity	↓→		↑	↑	↑	↓	↓
Diabetes							
Type 1	↓	→	↑		↑	↓→	↓
Type 2		↓	↑→	↑			↓
Hypertension							
- Hypertrophy		↑→			↑	→	→
+ Hypertrophy		↑→	↓		→	↓	↓
+ Dysfunction			↓		→	↓	↓
Heart failure							
Coronary*	↓	↓	↑		↓→	↓	↓
Idiopathic	↑	↓→	↓	↓	↓→	↓	→

Table 1.4: Derangement in myocardial substrate metabolism. Pathologic states mediate damage by alterations of substrate availabilities to the myocardium. GU: glucose uptake, FAU: fatty acid uptake, TG: triglycerides, PCr/ATP: phosphocreatine/ATP, ↑: increased, ↓: decreased, →: normal. * non-ischemic, non-dysfunctional regions. Table from Iozzo, 2010.

We will now focus on the last component contributing to cardiac energy metabolism, i.e. the differences between males and females. Please note that this section will only cover the effects on cardiac metabolism as sex differences in pharmacokinetics and dynamics is a vast field and lies outside the scope of this review.

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1.8 Effects of sex hormones

Dr. Sliwa points out that it is important to distinguish between sex and gender, even in molecular studies. Sex is the biological variations between men and women, i.e. the relative differences of sex hormones and different X and Y chromosome gene expressions (Regitz-Zagrosek, 2012). Sex steroids cause a wide variety of genes to respond in a sexually dimorphic way. For example, estrogens can cause the up- or down-regulation of genes in especially males, while the same is true for androgens. By contrast, gender encompasses the social and cultural differences between men and women, i.e. stress- and lifestyle- associated differences. Factors such as acceptance of invasive procedures, nutritional choices and gender-based exercise behavior have important consequences for gender-specific disease states (Regitz-Zagrosek, 2012). Although this review acknowledges the important role of gender in CVD, the focus will be solely on sex as the biological actions of sex steroids can be independent regulators in cardiac metabolism and function (Wittnich *et al.*, 2013).

The tenacious nature of sex differences in the clinical presentation of HF leads to the conclusion that a lot may be due to fundamental sex differences in cardiac physiology. Multiple clinical- and animal-based studies have therefore set out to investigate such differences in resting cardiac function (Ben-Sira & Sagiv, 1997; Beyer *et al*., 2001; Capasso *et al*., 1983; Convertino, 1998; Haykowsky *et al.*, 1998; Leblanc *et al*., 1998; Lerner & Kannel, 1986; Merz *et al*., 1996; Younis *et al.,* 1990). However, some of the data generated were contradictory and led to the conclusion that significant results were reflective not of inherent performance differences, but rather due to load differences. As a result some investigated isometric and isotonic contractions while controlling for pre- and afterload and found that there are no inherent sex differences (Capasso *et al.*, 1983). Thus the end results do not differ, but in essence it comes down to *how* the heart achieves its end parameters (Wittnich *et al.*, 2013). To further elucidate this, research also focused on sex differences in terms of cardiac excitation-contraction coupling and associated Ca^{2+} handling (Parks & Howlett, 2013). For example, comparative research work concentrated on contractile functional differences (Curl *et al*., 2001;

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Farrell *et al*., 2010; Howlett, 2010; Schwertz *et al.,* 2004; Vizgirda *et al*., 2002) and ovariectomy studies reported on Ca^{2+} transients, myofilament, sarcoplasmic reticulum (SR) Ca^{2+} sparks, SR Ca^{2+} content, Ca^{2+} current, diastolic Ca^{2+} and Na^{+}/Ca^{2+} exchanger (NCX) currents (Bupha-Intr *et al*., 2007; Fares *et al*., 2012; Kravtsov *et al*., 2007; Ma *et al*., 2009; Wattanapermpool & Reiser, 1999). It is clear that estrogen is not solely responsible for all such discrepancies. However, its unique role cannot be overlooked as it responds to signals of environmental, genetic and non-genetic origin (Konhilas & Leinwand, 2007). For the purpose of this review, the focus will not be on electrical and contractile effects of estrogen, but rather on its impact on metabolism, e.g. why healthy young human females utilize less glucose and more oxygen than age-matched male counterparts (Peterson *et al.*, 2007).

Skeletal muscle studies showed that estrogen decreased GO, gluconeogenesis and glycogenolysis, while it elevated FAO (Campbell & Febbraio, 2001). If this applies to the myocardium then it means that females rely more on FAO together with higher myocardial oxygen utilization. This reflects previous findings to explain the "double-edged sword" theory that follows estrogens' role in the heart (Wittnich *et al.*, 2013). Here estrogen offers advantage under well-oxygenated and perfused states, while the opposite is true during situations of oxygen deprivation where glucose utilization predominates (more ATP per oxygen).

Studies done on PPAR-α knock-out animals where CPT-1 was inhibited (Djouadi *et al.*, 1998) and where lipoprotein lipase (LPL) was overexpressed (Nöhammer *et al.*, 2003) demonstrate that females are better adapted to utilize FA and that they are also protected against increased FA levels compared to males (Konhilas, 2010). This ties in with data that show that larger VLDL particles with more triglycerides are secreted by female livers (Magkos *et al.,* 2007). In addition, VLDL production is higher in women but also VLDL-TG clearance (Mittendorfer *et al*., 2003), meaning that basal lipolysis is upregulated providing increased plasma FAs for utilization (Nielsen *et al.*, 2003; Spriet, 2002). This explains the higher LPL activity that is typically found in women (Farese *et al*., 1991; Spriet, 2002).

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Interestingly, LPL activity in adipose tissue and plasma is decreased by estrogen (Price *et al.*, 1998; Urabe *et al.*, 1996).

Estrogen also phosphorylates and thereby activates AMPK in many tissues, including adipose and the myocardium (Arad *et al.*, 2007; D'Eon *et al.*, 2005). AMPK acts to increase FA uptake and oxidation and also enhances glucose transport and glycolysis. It seems plausible that during stressful conditions, females take advantage of this metabolic state to increase circulating levels of fuel substrates. Estrogen also phosphorylates and activates the insulin-like growth factor 1 signaling pathway (Kahlert *et al.*, 2000; Richards *et al.*, 1996). PI3-K is activated by this pathway together with the downstream phosphorylation of AKT-1, a central regulator of glucose metabolism (Kandel & Hay, 1999). Conversely, estrogen can decrease glucose utilization in the myocardium by upregulating nitric oxide synthase (NOS). With NO overproduction leading to a decrease in AMPK-mediated GLUT-4 cell surface translocation via the second messenger cyclic guanosine monophosphate (Sharkey *et al.*, 1999; Skavdahl *et al.*, 2005). Estrogen is also the sole modulator known to affect ATP synthesis at complex 1 within the ETC (Rattanasopa *et al.*, 2015). It is not known how estrogen controls this balancing act in terms of cardiac energy metabolism and even less data exist regarding how results are affected in various pathological situations in patients with different clinical characteristics. To complicate matters even further, estrogen is not the only mediator in females although it dominates the follicular phase of the menstrual cycle. However, during the luteal phase progesterone plays a more predominant role.

Evidence is mounting that the protective effects of both estrogen and progesterone are rapid and non-genomic (Pang, Dong, & Thomas, 2015). Estrogen has received most attention while studies on the effects of progesterone are limited so far. However, some found that physiologically high levels of progesterone in both males and females are associated with increased CHF prevalence (Nilsson *et al.*, 2009). Mechanistic studies of progesterone revealed contradictory findings owing to supraphysiological levels tested and the nature of diseased tissue studied (Cheng *et al.*, 2012;

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Nakamura *et al.*, 2007). These studies reported on Na^+ efflux, Ca^{2+} uptake, action potential duration (Mendoza & De Mello, 1974) and force of contraction (Sitzler *et al.,* 1996). The autonomic function has also been implicated in progesterone studies, i.e. systole duration is reduced upon progesterone exposure (Fuenmayor *et al*., 2000; Re *et al.*, 1986). The first clues of progesterone's role in energy metabolism only emerged more recently, i.e. mediating its effects via the progesterone receptor-α, to subsequently activate PI3-K/AKT-1 and MAPK in addition to increased eNOS activity and NO production (Pang *et al.*, 2015; Simoncini *et al.*, 2004).

This concludes the underlying pathology section of this review. The reader will note that there is no set understanding of AHF specific pathology, the best that can be done is to review the pathologic mechanisms of underlying etiologies. At present the nature of the "switch" that determines whether the heart will develop CHF, ADHF or acute *de novo* AHF remains unclear.

The focus will now shift to current biomarkers and generic treatment of AHF, where after the emphasis will move to metabolism-derived therapies.

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1.9 Treatment of acute heart failure

1.9.1 Biomarkers

Despite the alarmingly poor understanding of its pathology, there has been a rapid accumulation of knowledge and understanding of the clinical presentation and characterization of AHF during the last few years (investigation into different characteristics between AHF and CHF patients only became "popular" since 2005). The increasing number of patients, the cost and sheer burden on health systems demand earlier diagnosis, improved risk stratification and treatment that is more cost effective. Moreover, the acute nature of this condition means that utilization of biomarkers remains a very useful tool for improved diagnosis (Maisel & Choudhary, 2012).

Research efforts reflect this as hundreds of articles were published in this regard during the last few years (started gaining momentum from around 2007). Current biomarkers include BNP, N-terminal of the prohormone brain natriuretic peptide (NT-proBNP), cystatin C, neutrophil gelatinase-associated lipocalin (NGAL), CRP, urinary albumin/creatinine ratio (UA/CR), galectin-3, suppression of tumorigenicity (ST2), L-type FABP (L-FABP), β-trace protein (β-TP), kidney injury molecule-1 (KIM-1), procalcitonin, surfactant proteins, s100-B protein, adrenomedullin (MR-proADM),creatinine, blood urea nitrogen (BUN), IL-6, IL-10, IL-18, and TNF-α (Damman *et al*., 2012; Di Somma *et al.*, 2012; Maisel & Choudhary, 2012; McCullough & Jefferies, 2015; Teichman *et al*., 2015). Although the use of biomarkers aims to achieve fast diagnosis, prognosis, therapeutic solutions and rapid discharge rates, a recent review reminded of the importance of patient history and physical examination (Schuetz *et al.,* 2015). The significance of a patient's overall *gestalt* (unified whole), rather than blind adherence to laboratory results was also emphasized. Patients suffer from hospital-acquired venipuncture, anemia and worrying hemoglobin levels as a result of bungles and repeats of "daily labs" (daily sample [blood, urine etc.] collections and ensuing laboratory testing). In addition, the test methodology, limitations, variations, interferences, false negatives and/or false positives are completely intertwined and can sometimes be misunderstood, leading to inaccurate conclusions.

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Thus it is the clinician's responsibility to make a humane and educated treatment decision based on all research and observations.

1.9.2 Current treatments

As for any acute onset event, diagnosis and treatment is mostly carried out in parallel (Table 1.5). During this process the close monitoring of patients is of outmost importance as any change in condition may require rapid implementation of a different treatment regimen. For further treatment of suspected/confirmed AHFS, the immediate goal is to improve symptoms and to stabilize the hemodynamic condition. Equally important is the pre- and post-discharge care of such patients (McKelvie *et al.*, 2012; McMurray *et al.*, 2012; Nieminen *et al.*, 2005).

AHF patients are treated with basic therapy consisting mainly of loop diuretics, vasodilators, opioids and oxygen (Table 1.6). However, the generic nature of such interventions show that current therapies are not tailored/designed specifically for AHF. Alternative treatment modalities were recently proposed but were generally unsuccessful as it did not lower re-hospitalization rates or improve patient outcomes (Hummel *et al.*, 2015). However, the crucial issue remains the ongoing failure to develop AHF-specific therapies

Here a key issue is patient heterogeneity and plurality in terms of AHFS (Felker *et al.*, 2010; Voors & Veldhuisen, 2012). There are similar signs/symptoms from a cluster of related diseases and yet this umbrella term contains subdivisions wherein parameters present differently. Thus it is unrealistic to expect one treatment to suit all. In addition, background or "standard therapy" varies extensively among studies – if it is indeed reported and it should be borne in mind that AHF treatment is routinely adjusted depending on patient progress. Natural disease history is also rarely reported and the limited information makes it hard to perform comparative studies. The inclusion criteria for studies are also variable, qualitative and subjective and hence the diagnosis often varies between hospitals, regions and studies. For example, inclusion of patients in trials that are not actually suffering from AHF dilutes the potential effects of the drugs being tested. Knowledge, understanding

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and implementation of intervention and treatment timing, duration and "therapeutic windows" are also suboptimal as are experimental endpoints. The choice of hard and/or soft endpoints and the general consensus around primary endpoints is of course also a critical study design issue. Regulatory requirements and approval of drugs and studies also greatly affect the design of international studies.

Initial assessment	
History and physical examination, blood pressure, respiratory frequency, chest x-ray, ECG, echocardiography and/or NT-proBNP, blood-gas analysis, laboratory tests (including troponin, complete blood count, electrolytes, renal parameters, TSH, albumin, blood glucose).	
Simultaneous specific assessment	**Acute treatment**
Hypoxemia, hyperventilation, hypoventilation	Oxygen; non-invasive or invasive ventilation
Life-threatening arrhythmia or bradycardia	Electric cardioversion, external pacemaker
Blood pressure <85 mmHg or shock	Inotropic agents, vasopressors, mechanical circulatory support
Acute coronary syndrome	Coronary reperfusion, antiplatelet agents, anticoagulation
Acute mechanical cause/severe valvular disease	Cardiac surgery, pericardial puncture, Interventional treatment

Table 1.5: Parallel assessment and treatment of acute heart failure. Acute diagnostic assessment is based on the unique clinical presentation of each patient. ECG: electrocardiography, NT-proBNP: N-terminal of the prohormone brain natriuretic peptide, TSH: thyroid stimulating hormone. Table from Hummel *et al.*, 2015.

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Pharmacological therapy: **Acute management**	Other pharmacological therapies are used like anticoagulants and vasopressin V2-receptor antagonists. However these come with contraindications and uncertain or extreme adverse side effects.
Oxygen: Utilized to treat hypoxemia (SpO_2 <90%). Important to prevent end-organ dysfunction and multiple organ failure.	
Diuretics: Administered in the presence of fluid retention symptoms (peripheral and/or pulmonary edema). This leads to a decrease in extracellular fluid volume, plasma, total body water and sodium. This ultimately leads to reduced left and right ventricle filling pressures in addition to reduced edema.	**Pharmacological therapy:** **After stabilization**
	ACE-inhibitors: Achieves its hemodynamic effect through decreased AII and bradykinin level increases. This results in general and total decreases in peripheral vascular resistance.
Opiates: Administered to patients with dyspnea associated anxiety and distress. They induce venodilation (reduced preload), mild arterial dilation and also serve to reduce sympathetic drive or heart rate.	Mineralocorticoid (aldosterone) receptor antagonists and Digoxin (anti-arrhythmia drug): Advised on the condition that there are no contra-indications involved.
Vasodilators: Of most use to hypertensive patients. They increase stroke volume and lower preload and afterload.	Beta-blockers: Can limit infarct size and reduce arrhythmias and pain.
Inotropes: Administered as a last resort and with much caution. These patients have drastically low cardiac output, to the point of hypoperfusion of vital organs. These are hypotensive “shocked” patients.	**Non-pharmacological/non-device therapy**
Vasopressors: Epinephrine and norepinephrine are administered only in case of emergencies (to sustain life) where a patient is in life threatening hypoperfusion after diuretics and inotropes have failed. They redeploy the cardiac output from the extremities to the vital organs and raise the blood pressure.	Non-invasive ventilation such as NIPPV and CPAP do relief dyspnea and it also improves some physiological measurements such as oxygen saturation. However if a patient is experiencing symptoms such as respiratory failure (acidosis and hypoxemia) and diminished consciousness, then endotracheal intubation must be utilized. Mechanical circulatory support devices include intra-aortic balloon pumps to support circulation before surgical corrections and ventricular assist devices. Ultrafiltration (venovenous isolated) can be used to remove fluid when patients are resistant and/or unresponsive to diuretics.
Dopamine: Have inotropic and vasoconstrictor effects at high doses and at low doses has renal arterial vasodilator (selective) effects.	

Table 1.6: Untailored treatment for acute heart failure (AHF). Treatment of AHF can be divided into two categories, namely pharmacological (acute management and after stabilization) and non-pharmacological/non-device therapy. SpO_2: arterial oxygen saturation, ACE: angiotensin converting enzyme, AII: angiotensin II, NIPPV: non-invasive positive pressure ventilation, CPAP: continuous positive airway pressure. Table adapted from McKelvie *et al.*, 2012; McMurray *et al.*, 2012; Nieminen *et al.*, 2005.

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1.10 Movement towards metabolic-mediated therapies

The relative contribution of fuel substrates to ATP production is tightly controlled/regulated and this plastic process includes the interdependence of substrates. Here shifts in substrate selection and utilization can occur rapidly and multiple times a day depending on changing situations, availability of substrates and/or hormone changes.

Cardiac function and efficiency is strongly reliant on the relative contributions of FAO. For example, if a pathologic state disrupted such homeostasis it would alter cardiac metabolism, function and efficacy into a spiral towards cardiac injury. This is not a new concept and the first description of the so-called Randle effect was already published in 1961 (Garland *et al.*, 1963; Randle *et al.*, 1963; Randle *et al.*, 1964). Thus an intriguing question emerges regarding the failure of hemodynamically-related drugs (previously discussed) and whether targeting cardiac metabolism/energetics may instead provide novel therapeutic opportunities.

Such ‘’metabolic’’ drugs mainly fall into one of four categories based on specific target sites. The first group is targeted at the availability of circulating FAs: glucose-insulin-potassium therapy (GIK), β-adrenoceptor antagonists and PPAR-α agonists. The second group targets the uptake of mitochondrial FA: etomoxir (CPT-1 inhibitor), perhexiline (CPT-1 inhibitor) and malonyl-CoA decarboxylase inhibitors. The third group targets mitochondrial FAO: trimetazidine (TMZ) (discussed next) is a 3-KAT inhibitor and ranolazine (multiple sites of action). The last group targets the PDH complex and GO: PDHK inhibitors (Jaswal *et al*., 2011).

The reader will notice the focused attention on only glucose and FA metabolism. This is because in the absence of pathology, 60-80% of ATP production is generated from FAO, while the remainder is derived from carbohydrate (glucose and lactate) and also from ketone bodies (Bing *et al.*, 1954; Neely & Morgan, 1974; Opie, 1968; Opie, 1969). A critical consideration to keep in mind is that the aim is not to completely shift or force cardiac metabolism to a sole substrate, but rather to restore homeostasis and metabolic plasticity.

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1. 11 Trimetazidine

Studies investigating the therapeutic use of metabolic moderators have yielded inconsistent data, although it emerges that TMZ holds the most clinical promise (Tuunanen & Knuuti, 2011) as it is the most prescribed metabolic modulator and is available in more than eighty countries around the globe (Folmes *et al*., 2005). This anti-anginal drug is a partial inhibitor of the last enzyme of FAO, i.e. 3-KAT. TMZ shifts cardiac metabolism to GO via the Randle cycle (Kantor *et al*., 2000; Lopaschuk *et al*., 2003). However, it has lately emerged that TMZ's positive effects can also be due to other mechanisms (Parkhomenko & Gurjeva, 2014) that include an impact on inflammatory and endothelial function, proton reduction and effects on the MPTP. Thus the beneficial effects of TMZ are far reaching as further discussed below.

TMZ showed positive clinical effects in patients with recurrent/stable angina where they showed improved exercise test duration, total workload, time to 1 mm ST segment duration and time to angina onset. Such patients also required decreased nitrate consumption and exhibited a lower number of angina attacks (Hu *et al.,* 2011; Ribeiro, 2012; Ruzyllo *et al.*, 2004; Szwed, 2004). Within the context of ischemic reperfusion injury, TMZ displays effects on signaling pathways, ROS (Kutala *et al.*, 2006), opening of the MPTP, the permeability of the mitochondrial membrane, OS, caspase-3 activity, apoptotic and necrotic cell loss (Argaud *et al.*, 2005), inflammatory cascades (Kuralay *et al*., 2006), neutrophil accumulation and neutrophil-mediated injury (Tritto *et al.*, 2005; Williams *et al.,* 1993), pro-survival AKT-1 enzyme, p38 MAPK (Khan *et al.*, 2010) and eNOS (Di Napoli *et al.*, 2007). Patients present with reduced dispersion of the Q-T interval and heart rate variability and a large myocardial infarction registry reported improved survival and reduced new incidents of myocardial infarction with TMZ (Kim *et al.*, 2013). The employment of percutaneous angioplasty that accompanies myocardial ischemia treatment is also reduced (Polonski *et al*., 2002) with TMZ treatment. Patients show reduced pressure overload fibrosis via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-ROS-connective tissue growth factor signaling pathway

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(Liu *et al.*, 2010). Here TMZ did not act via the expected FAO reduction, but rather by decreased proton production, i.e. decreasing glycolysis, recoupling glycolysis and GO and also by tempering acidosis (Liu *et al*., 2002; Liu *et al*., 1996; Saeedi *et al.*, 2005). CAD patients treated with TMZ displayed a 30% greater survival rate versus controls (El-Kady *et al*., 2005) and here it seems the inflammatory system is targeted with reduced CRP, TNF-α and nitrite levels (Kuralay *et al.*, 2006). In patients with idiopathic dilated cardiomyopathy improvements in walk tests and left ventricular contractility were noted, i.e. displaying lower B-type BNP levels (Zhao *et al.*, 2013). Other studies also found reduced FAO, increased GO and favorable changes in ejection fraction, high-density lipoproteins (HDL) and insulin resistance was observed (Tuunanen *et al.*, 2008; Zhao *et al.*, 2013).

Beneficial effects of TMZ have been found for unspecified, ischemic and non-ischemic HF. Patients experience improved quality of life, New York Heart Association (NYHA) class, exercise tolerance, metabolic shift (FAO to GO), PCr/ATP ratio, whole body insulin sensitivity, ejection fraction, left ventricular diastolic function, left ventricular end diastolic volume, regional myocardial wall motion (at rest and stressed), functional capacity and endothelial function. Moreover, reduced mortality, hospitalizations and BNP levels have been documented (Gao *et al*., 2011; Jaswal *et al.*, 2011; Ribeiro, 2012; Tuunanen & Knuuti, 2011). There is also evidence that TMZ reversed left ventricular remodeling and limits the inflammatory response (Di Napoli *et al*., 2005; Fragasso *et al.*, 2006). Of note, a study on HF in rabbits found that TMZ's benefits where not due to expected FAO outcomes, but instead due to mitochondrial cardio protective effects. Here it lowered electron leaks from complex 2 in the ETC, improved the function of complex 1, uncoupled mitochondrial NOS (reducing ROS) and decreased the likelihood of MPTP opening (Dedkova *et al*., 2013). Thus the picture emerges that TMZ acts on multiple levels and thus raises the question whether it may offer therapeutic value for AHF patients.

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1.12 Summary

ADHF is the most common primary diagnosis in heart disease patients admitted to African hospitals. Moreover, African women suffering from AHFS are younger, with significant stress on the 20-29 year old group and also more prone to suffer from acute *de novo* HF. This type of AHF is also associated with increased risk of death compared to other forms. In addition, co-morbidities such as diabetes and obesity further complicate the picture. In light of this bleak scenario the optimization of cardiac metabolism and especially TMZ emerges as a novel and worthy therapeutic option to investigate for the treatment of AHF.

1.13 Hypothesis and aims

For this study, we hypothesized that TMZ, a partial FAO inhibitor, offers cardio protection during AHF under normal and obese-related diabetic conditions and we further propose that the estrous cycle will influence such cardio protection.

The aims of this study were: (1) to establish a *de no* AHF mouse model; (2) to test whether simple filtering could replace the gold standard dialysis method; (3) to identify and establish the four phases of the estrous cycle in the female mice; and (4) to evaluate the therapeutic value of TMZ under normal and obese-related diabetic conditions in both male and female mouse hearts subjected to a unique *ex vivo* model of AHF. We also tested the influence of the different phases of the estrous cycle (follicular and luteal) on the efficacy TMZ and assessed whether there were any sex differences in terms of TMZ treatment.

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Chapter 2: Materials and methods

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2.1 Animals and ethics statement

Animals were treated in agreement with the Guide for the Care and Use of Laboratory Animals of the National Academy of Science (National Institutes of Health publication No. 85-23, revised 1996). This study was executed with the approval of the Animal Ethics Committees of Stellenbosch University (South Africa) (Addendum A).

For the experimental procedure, 102 male and female diabetic (*db/db*) mice (12-14weeks) and their lean non-diabetic littermates (*db/+*) were purchased from Jackson Laboratories (Bar Harbor, Maine, United States). Animals were divided into 17 groups as set out in Table 2.1. Mice were housed six per cage in the animal housing facility under standard conditions and had *ad libitum* access to water and standard chow.

Allocation of Animals			
Filter versus dialysis experiments		**Filter**	**Dialysis**
Lean males		n=6	n=6
Acute heart failure experiments	**Control**	**CAHF treated**	**RAHF treated**
Obese males	n=6	n=6	n=6
Obese females	n=6	n=6	n=6
Lean males	n=6	n=6	n=6
Lean females (follicular phase)	n=6	n=6	n=6
Lean females (luteal phase)	n=6	n=6	n=6

Table 2.1: Group allocations. CAHF: critical acute heart failure, RAHF: recovery acute heart failure.

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2.2 Vaginal cytology

The estrous cycle of females (12-14 weeks old) was determined by vaginal smear cytology or "wet smears" as previously described (Caligioni, 2009; Long & Evans, 1922; Marcondes *et al.,* 2002; Wood *et al*., 2007). Because group-housed female mice do not display reliable regular estrous cycling (MacDonald *et al*., 2014; Whitten, 1958), bedding from young male mice were placed in the female cages a minimum of 5 days prior to performing estrous phase determination. Vaginal secretion was collected by inserting a pipette tip filled with 10 µl normal saline into the vaginal opening, taking care not to insert it too deep as to cause harm to the female. The saline was injected and the vaginal flush was collected in the same tip which was then transferred to a clean glass slide (Labstar frosted microscope slides, Lasec Laboratory Solution Provider, Cape Town, South Africa). The unstained material was viewed under a light microscope (YS 100, Nikon Co., Chiyoda, Tokyo, Japan) (without use of condenser lens) under 4x, 10x and 40x objective lenses. Three distinct cell types were present, namely epithelial cells (round and nucleated), cornified epithelial cells (irregular in shape, absence of a nucleus) and leukocytes (black, small and round). The ratio or proportion of such in the vaginal flush was used to determine estrous cycle phases. Proestrus was characterized by the predominance of epithelial cells; estrus by the majority of cornified epithelial cells; metestrus by the presence of a mixed cell type dominated by leukocytes and diestrus by the bulk of leukocytes.

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2.3 Testing filter versus dialysis

Due to the sensitive nature of the *ex vivo* perfused mouse heart, perfusates containing bovine serum albumin (BSA) should be purified. Traditionally, researchers utilize the dialysis method but this is an expensive and time consuming method. In light of this, we aimed to test whether filtering the perfusates would deliver comparative results.

We excised (intramuscular injection of ketamine (100 mg/kg) and xylazine (5 mg/kg) (obtained from the animal facility manager) and perfused 12 lean male non-diabetic (*db/+*) mouse hearts at constant 100 cmH_2O perfusion pressure for 30 minutes with Krebs-Henseleit buffer (Addendum B) with 10 mM/L glucose (#G7528, Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States) and 1.2 mM/L palmitic aid (#P0500, Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States) bound to 3% BSA (BSA Fraction V, Roche Ltd., Basel, Switzerland) as previously described (Lopaschuk & Barr, 1997). The buffer of the dialysis group (n=6) was dialyzed (#68100, SnakeSkin Dialysis Tubing [23µm], Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States) overnight according to the stipulated protocol while for the filter group (n=6) we filtered the buffer through a low protein binding filter (Durapore Polyvinylidene fluoride [PVDF] 0.22 µm, Merck Millipore Co., Darmstadt, Germany) using the plastic structure of a bottle top vacuum filter (#431118, Corning Inc., New York, United States) and a vacuum pump (2522C-02, Welch, Niles, Illinois, United States) on the day of perfusion.

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2.4 Acute heart failure retrograde Langendorff heart perfusion

The mouse *ex vivo* Langendorff retrograde perfusion model of *de novo* AHF was adapted from a rat model previously set up (Deshpande *et al.,* 2014; Deshpande, 2013; Deshpande *et al*., 2010; Opie *et al.,* 2010).

2.4.1 Perfusing mouse hearts

Male and female mice (immediately following estrous phase determination) were anesthetized by an intramuscular injection of ketamine (100 mg/kg) and xylazine (5 mg/kg), the hearts rapidly excised and mounted on a cannula connected to the modified Langendorff retrograde perfusion model - flow from the stabilization phase was immediately commenced. The left atrial appendage was cut off and a deflated self-made balloon constructed from cling wrap (connected to a distilled water [dH_2O]-filled catheter [Portex Jackson cat catheter with luer connection, 4F, Smiths Medical International Ltd., Kent, United Kingdom]) was placed into the left ventricle via the mitral valve. Using an insulin syringe connected to the pressure transducer (Stratham MLT 0380/D, AD Instruments Inc., Bella Vista, New South Wales, Australia) compatible with the PowerLab System ML410/W (AD Instruments Inc., Bella Vista, New South Wales, Australia), the balloon was inflated to fill the left ventricle. A pacer (self-made) was connected to the right atrial appendage of the heart and set to 400 bpm. A buffer filled reservoir - correlating to each phase - was raised around the excised heart to maintain the heart at a stable temperature of 37.5˚C. The PowerLab System was set to record the following functional parameters: left ventricular end diastolic pressure (LVEDP), left ventricular end systolic pressure (LVESP), left ventricular developed pressure (LVDevP) [calculated as LVESP-LVEDP], heart rate, rate pressure product (RPP) calculated as [heart rate x LVDevP] and $(dp/dt)_{max}$ (an index of myocardial contraction velocity) [calculated as pressure difference in mmHg/time in sec].

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2.4.2 Rationale of the model

Each of the three phases (discussed below) was maintained for 25 minutes, for a total protocol duration of 75 minutes. The model consists of three phases, namely (1) stabilization, (2) critical acute heart failure (CAHF), and (3) recovery acute heart failure (RAHF) (Figure 2.1). Each phase can be independently induced by cut-off stop cocks at the other reservoirs. The buffer for all three phases was based on standard mouse Krebs-Henseleit buffer, with deviations only in glucose and FA concentrations (Addendum B). All buffers were gently gassed with 95% O_2/5% CO_2 (#801068-RC-A, AFROX, Gauteng, South Africa) for 20 minutes prior to perfusion and for the duration of the experiment. The system consists of a series of double-jacketed glass reservoirs connected by piping. A water bath (HAAKE SC100 & S3, Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States) constantly circulates heat-controlled water through the outer jacket of the system to maintain the perfusate temperature in the reservoirs at 37.5 °C.

Stabilization phase:

This phase is solely to stabilize the heart and to achieve baseline functional parameters. It also functions to flush the heart of any residual blood that could introduce metabolites into the model, potentially affecting the function. This phase is perfused at 100 cmH_2O perfusion pressure with glucose (10 mM/L) as the sole substrate. Hearts that do not achieve a stable heart rate of 400 ± 20 beats per minute (bpm) and a left ventricular developed pressure of >20 mmHg were excluded from the study.

Critical acute heart failure phase (CAHF):

This phase mimics the state of hypotension that patients with AHF suffer and also aims to simulate the shift in substrate utilization (GO toward FAO) found with HF and under ischemic conditions. For this reason this phase is perfused at a reduced perfusion pressure of 20 cmH_2O and the glucose concentration is decreased to 2.5 mM/L, while introducing 1.2 mM/L FA (palmitic acid [the most

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abundant FA in human tissues] conjugated to 3% BSA) into the buffer (Oliveira et al., 2015). This phase was allowed to continue without any pacing in order for the heart to fail 'naturally' - a patient will not experience stable heart rates during this phase.

Recovery acute heart failure (RAHF) phase:

The perfusion pressure is restored back to 100 cmH_2O and the pacer (unchanged from 400 bmp setting during stabilization) is turned on again to mimic a recovering patient who has been hemodynamically stabilized. Glucose concentrations are also restored to 10 mM/L baseline condition, with the high FA content (1.2 mM/L) being maintained in the recovery buffer, to give the recovering heart a choice in terms of metabolic fuel substrate utilization during this recovery stage.

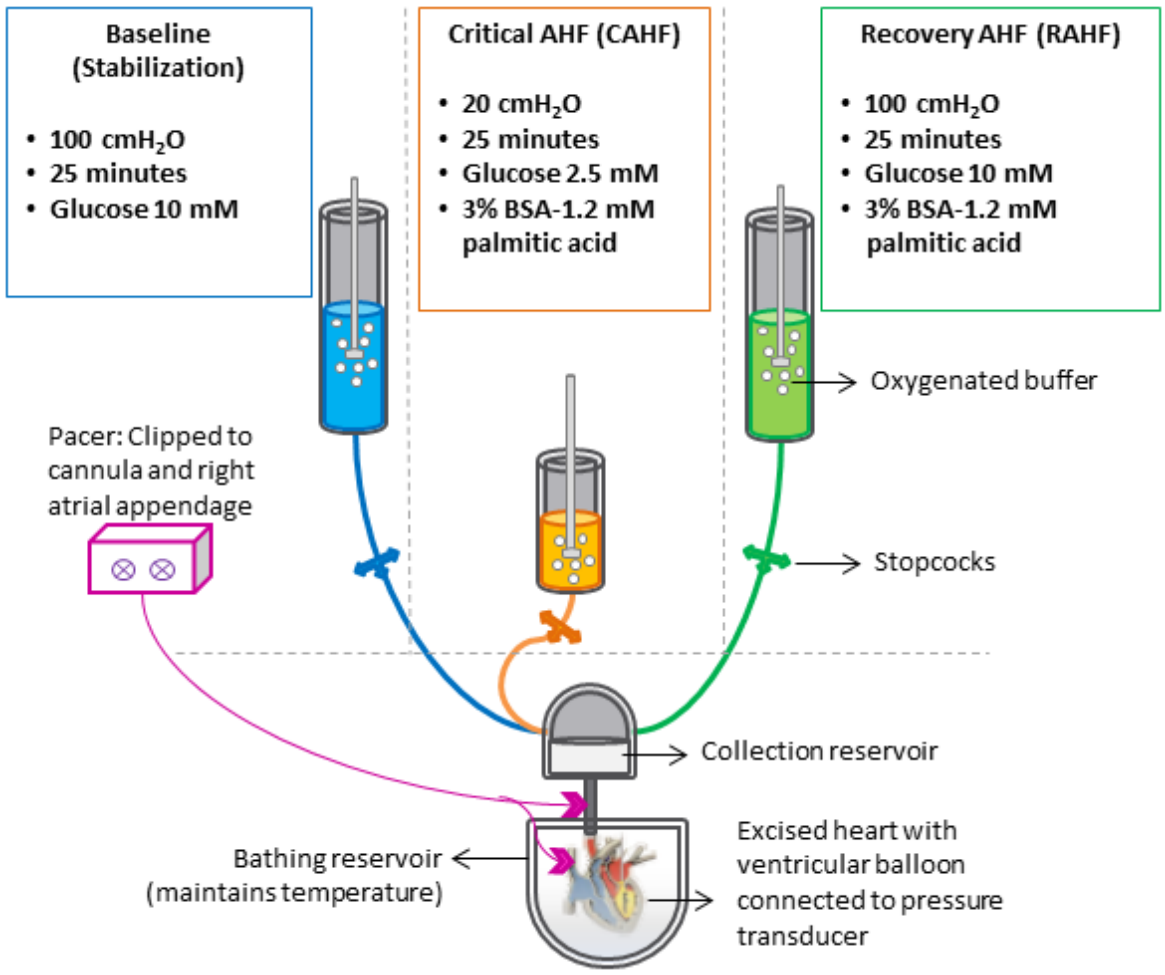

Figure 2.1: Schematic representation of the modified retrograde Langendorff model to induce acute heart failure (AHF).

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2.5 Treatment with trimetazidine

Control groups were allowed to go through the full protocol without any intervention. Treated groups received TMZ (5 µM) (#653322, Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States) in the perfusate in either the CAHF or RAHF stage for the full duration of the phase. The therapeutic dose was selected based on mean plasma TMZ concentrations in a clinical study (Barré *et al.*, 2003) and translated to an equivalent animal dosage as previously described (Reagan-Shaw *et al.*, 2008).

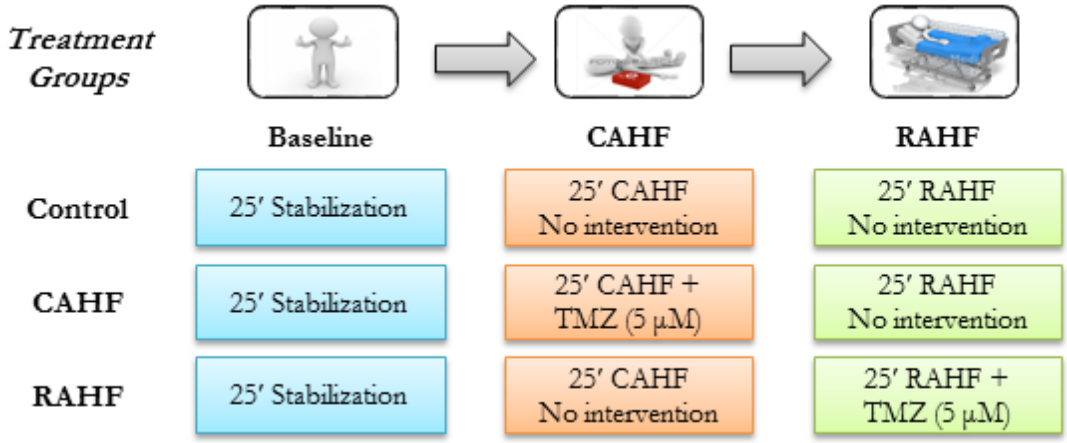

Figure 2.2: Schematic representation of treatment regime.

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2.6 Producing murine ventricular balloons for the Langendorff heart perfusion

Aortic retrograde perfusion in the Langendorff mode is a widely used technique in both academia and in the industry. Intrinsic rhythmic contraction can be maintained for hours in order to measure functional parameters of heart function by means of a dH_2O-filled balloon that is inserted into the left ventricle of the excised and mounted heart. Traditionally, the rat is employed for this technique and manufactured latex balloons are commercially available for hearts or alternatively it can be easily prepared by the researcher. This is, however, not the case when utilizing mice hearts for Langendorff perfusions as there are no commercially available balloons. Moreover, manufacturing a ventricular balloon that is small enough and made from a thin material that has adequate tensile and elastic properties has been a challenge (Miller & Wright, 2011), especially in South Africa. We therefore set out to manufacture our own mouse ventricular balloons that would: 1) be appropriately sized (including tubing); 2) be free from sharp edges (due to material bunching); 3) fill and form to the contours of the ventricle (highly flexible); 4) be made from a thin, highly compliant and non-elastic material that can accurately transmit functional data to the fluid in the balloon and 5) have high linear frequency response (appropriate transducer, tubing and attachments) (Liao *et al.,* 2012).

2.6.1 Optimization of ventricular balloons

We prepared and tested ventricular balloons constructed from multiple materials. Here latex-based balloons (constructed from condom tips and dental dams) were a) too thick and elastic (not in compliance with the requirements set out above) and b) when inflated yielded perfectly round balloons that would not fill nor transmit pressures from the bottom tip of the ventricle (inadequate ventricular filling). By contrast, balloons constructed from multiple different types of plastic sheeting yielded irregular shaped balloons with sharp edges (non-compliant material) that caused damage to the ventricle. We subsequently decided to adapt a fluid silicone method (Miller & Wright, 2011) where pins were dipped into molten caramelized sugar to form a stem and teardrop

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that was left to dry. This was then dipped into fluid silicone (silicone mold star 16, SM420 and SM421, amt composites, Maitland, Cape Town, South Africa) to form a silicone balloon over the candy and once dried was removed by placing it into warm water to melt the candy. Although the precise shape/size of such balloons was easily controlled, the balloon was still too thick and incompliant. An additional approach was thereafter assessed i.e. we utilized commercial food wrap (''cling film'') and prepared the balloons following the instructions of ADInstruments (http://cdn.adinstruments.com/adi-web/techniques/TN-BalloonCatheter.pdf). Although this resulted in an improvement on the previous plastic sheeting balloons, it still yielded irregular and sharp edged balloons.

2.6.2 Constructing ventricular balloons for mouse heart perfusions

For accurate measurement of ventricular pressures, the ventricular balloon (and tubing) should be small enough, void of any damaging corners, should occupy the full shape of the ventricle and must accurately relay cardiac pressures into the fluid contained within the balloon (Addendum C). We eventually found the best method of constructing murine ventricular balloons to be: 1) pre-stretched industrial grade cling film; 2) 5-0 silk suture replaced with "invisible thread" (available from any craft store) and 3) commercially available cat catheters (size F4) as a replacement for common plastic tubing.

The first step was to cut a small piece of cling wrap (approximately 3 x 3 cm). Using a size 13 knitting needle held upright with the point to the top, the cling wrap was slowly pulled 1-2 cm over the point of the needle. We found holding the cling wrap in close proximity to the needle shaft a good strategy as this helps to not only stretch the cling wrap to its maximum but also to "shape" it better. The cling wrap was then slowly and gently removed from the knitting needle. With the catheter attached to the syringe, degassed distilled dH_2O was sucked into the syringe while making sure there were no air bubbles in the catheter. The tip of the dH_2O-filled catheter was then placed into the cone shape, leaving a space of at least 5 mm between the tips of the cling wrap cone and the catheter. The

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invisible thread was then neatly wrapped and tied off around the catheter: we left a tail of thread at the back (syringe side) and started winding from the tip of the catheter upward for about 1 cm. The starting point of the "coil" was close to the tip of the catheter – max 1 mm distance. We knotted-off sufficiently to prevent leaks and winding should be done neatly to avoid repeated overlap of thread. There should be no openings between successive coils. In addition, the winding should also be tight enough to prevent leaks, however, winding too tightly could also pinch close the catheter. The resulting inflated and dH_2O filled balloon should not only be a teardrop shape, but more importantly there should be no sharp edges or corners on the balloon. There should be absolutely no air bubbles in the balloon or the catheter, if there is it can be removed by repeated inflating and deflating while flicking the catheter and/or syringe in the upright position. Throughout making the balloons, additional care should be taken not to kink the catheter as this influences the functional readings.

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2.7 Blood and tissue collection

Blood was collected from the thoracic cavity after excision of the heart and centrifuged (Spectrafuge 24D, Labnet International, Inc., Edison, New Jersey, United States) at 3000 rpm (828 x g) for 15 minutes in order to collect the serum. After the cessation of the RAHF phase the left ventricles of hearts were snap frozen in liquid nitrogen and all samples stored at -80˚C for future use.

2.8 Analysis

2.8.1 Evaluation of serum levels of estradiol and progesterone

Commercially available enzyme-linked immunosorbent assays (ELISA) were used to assess hormonal concentrations of estradiol (DE4399, Demeditec Diagnostics, Kiel, Germany) and progesterone (DEV9988, Demeditec Diagnostics, Kiel, Germany) in serum. The assay procedures were carried out as stipulated in the instructions provided. Both kits were solid phase ELISAs that are based on competitive binding principles. In short, the microtiter wells are coated with an antibody directed to each hormone, respectively. The unknown amount of hormone that is present in each sample competes with horseradish peroxidase conjugated estradiol or progesterone for binding to the coated antibody. The unbound conjugates are washed off after incubation, meaning that the bound peroxidase conjugate is reversely proportional to the hormone level in the sample. Addition of the substrate solution allows for colorimetric evaluation of hormone concentrations in the sample. Both kits contained standards from which to produce a standard curve in order to determine final hormone concentrations in the samples.

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2.8.2 Evaluation for protein expression levels

Protein determination for 3-KAT and PDH were performed by Western Blot analysis (refer to Addendum D for complete protocol). In short, left ventricular tissues from each treatment group were homogenized and resuspended in RIPA (modified RadioImmunoPrecipitAtion) buffer. After centrifugation, the whole fractions were used to determine the total protein concentration using the Bradford method (Bradford, 1976). Sample (six samples per group to make a total of 90 samples) preparations (50µg per well) were run on 12% 1 mm sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (SDS-PAGE), supplemented with 0.5% v/v trichloroethanol (TCE) (#T54801, Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States). The addition TCE to the gels allow us to utilize the Bio-Rad Stain-Free technology. The separated proteins were electrophoretically transferred to PVDF membranes (Trans-Blot Turbo Transfer System Transfer Pack, Bio-Rad Laboratories Inc., Hercules, California, Unites States) using the Bio-Rad Trans-Blot Turbo Transfer system V1.02 (Bio-Rad Laboratories Inc., Hercules, California, Unites States). Protein standards (#1610377 Precision Plus Dual Xtra Prestained and #1610396, Precision Plus Protein Unstained, Bio-Rad Laboratories Inc., Hercules, California, Unites States) were run in each gel. The blots were blocked in Tris-buffered saline-Tween-20 (TBS-T) supplemented with 5% fat free milk (Parmalat, Stellenbosch, South Africa). Blots were incubated overnight at 4°C with the various primary antibodies at 1:1,000 dilutions in TBS-T. Primary antibodies used were 3-KAT mouse (Anti-ACAA1, #Ab110289, Abcam plc., Cambridge, United Kingdom) and PDH rabbit (#2784 Cell Signaling Technology Inc., Danvers, Massachusetts, United States). Membranes were washed in TBS-T before incubation for 1 hour with either 1:8,000 anti-mouse IgG, HRP-linked secondary antibody (#7076S, Cell Signaling Technology Inc., Danvers, Massachusetts, United States) or 1:4,000 anti-rabbit IgG, HRP-linked secondary antibody (#7074S, Cell Signaling Technology Inc., Danvers, Massachusetts, United States) diluted in TBS-T. Blots were treated with Enhanced Chemi-Luminescence (ECL) (Bio-Rad Clarity Western ECL substrate, Bio-Rad Laboratories Inc., Hercules, California, Unites States)

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reagents and the required proteins were detected using the Bio-Rad Chemidoc MP Imaging system (Bio-Rad Laboratories Inc., Hercules, California, Unites States). Quantification of protein expression was analyzed using Image Lab Software Version 5.0 (#170-9690, Bio-Rad Laboratories Inc., Hercules, California, United States). Blots were normalized to total protein as stipulated in the Bio-Rad Stain-Free technology protocols that is included in Addendum D.

2.9 Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Data was analyzed using either a one-way or two-way analysis of variance (ANOVA) where appropriate, followed by a Bonferroni *post-hoc* test (GraphPad Prism v5, San Diego, California, United States). A value of $p<0.05$ was considered significant.

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Chapter 3: Results

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3.1 Characterizing the estrous cycle

We initially set out to determine whether the different stages of the estrus cycle could be identified, namely the follicular (proestrus and estrus) and luteal phases (metestrus and diestrus). Thereafter we assessed whether such phases corresponded with the expected hormonal fluctuations of estradiol and progesterone. Here the follicular phase should be characterized by high estradiol levels, whereas in the luteal phase, progesterone levels are expected to predominate.

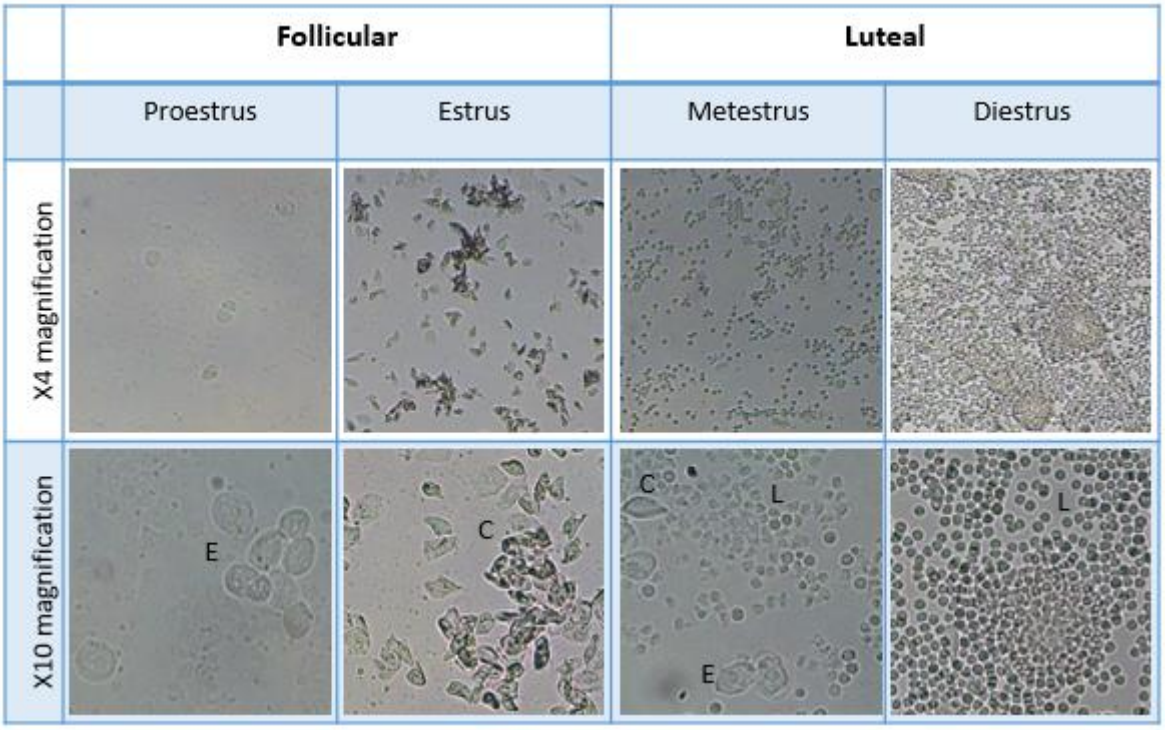

Figure 3.1: Stages of the estrus cycle characterized by vaginal cytology. The phases are characterized by the ratio of cells that are present. Indicated are nucleated epithelial cells (E), cornified squamous epithelial cells (C) and leucocytes (L). Images represent the vaginal flush of lean females under 4x and 10x magnification.

Slides prepared from mice in the proestrus phase showed a predominance of nucleated epithelial cells and occasionally some cornified cells and/or leukocytes were present (Figure 3.1). In the estrus phase there was a predominance of cornified squamous epithelial cells that are easily identifiable by its irregular shape and granular cytoplasm. The metestrus phase is characterized by the presence of mixed cell types although a predominance of leukocytes occurs, while the diestrus phase is

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completely dominated by exceptionally dense depositions of leukocytes (Figure 3.1). While all four phases were detected in lean females, obese females did not undergo any follicular phases.

Average levels of estradiol and progesterone in serum of lean female mice showed no statistical differences for any of the phases (Figure 3.2). Obese males and females and lean male sera were also tested but did not show any statistical significant differences (data not shown).

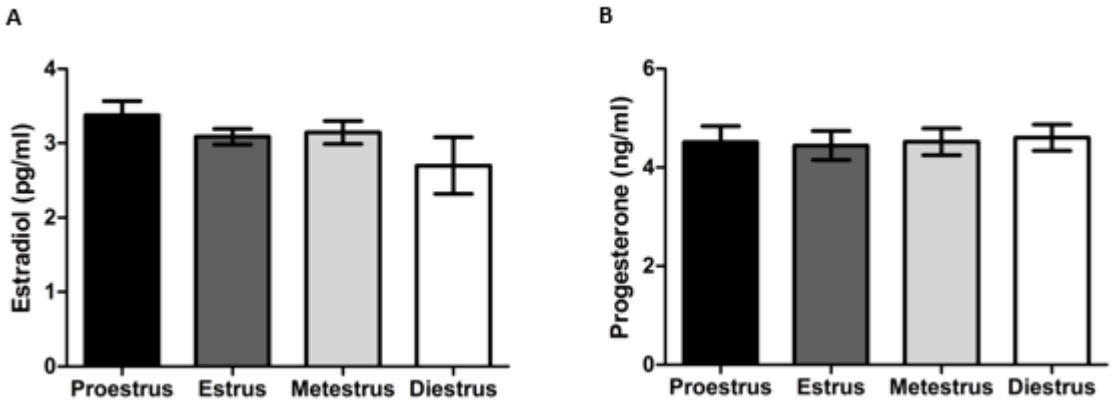

Figure 3.2: Average estradiol and progesterone levels in serum of lean female mice throughout the estrus cycle. (A) Estradiol (B) Progesterone (n=6).

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3.2 Comparing the filter and dialysis method

The dialysis method to prepare conjugated fatty acids: serum albumin preparations are routinely used for heart perfusion studies. However, this method is time consuming (requires overnight stirring) and also costly. We therefore set out to test whether a simplified protocol (filtering) could achieve the same results and thus potentially replace the classic dialysis method.

For the duration of the experiment, the dialysis group displayed increases in both RPP and $(dp/dt)_{max}$ ($p<0.05$) versus the filter group that stayed constant (Figure 3.3 A). Moreover, at the cessation of the experiment the dialysis group exhibited a higher $(dp/dt)_{max}$ ($p<0.05$) (Figure 3.3 B) and a moderately lower heart rate (Figure 3.3 C) ($p<0.01$) compared to the filter group. The dialysis group also showed a higher LVDevP, although this was not statistically significant (Figure 3.3 D). A decrease in LVEDP for the filter group was noted between the groups at the last time point (Table 3.1). No differences in LVESP were observed (Table 3.1).

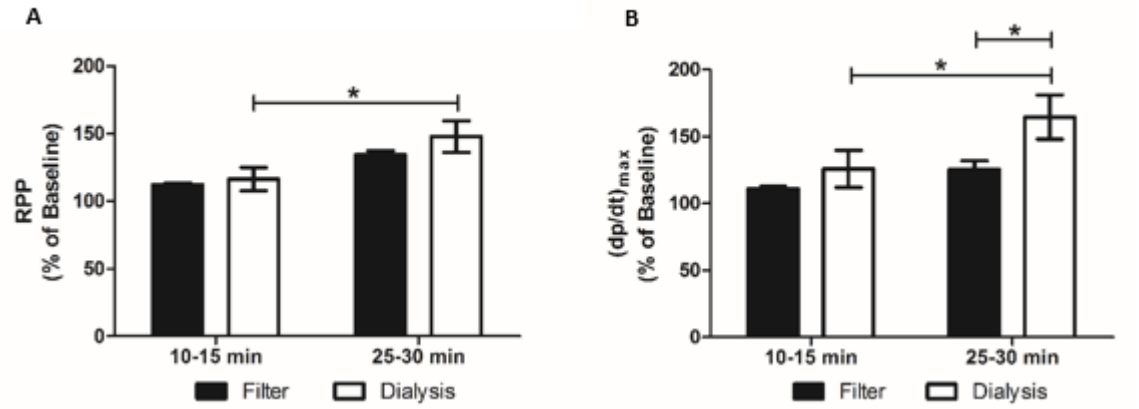

Figure 3.3: Characterization and comparison of the filter and dialysis method in lean male mice. (A) RPP (B) $(dp/dt)_{max}$ (n=6). Significance is expressed as *$p<0.05$ versus dialysis 25-30 min. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, min: minutes.

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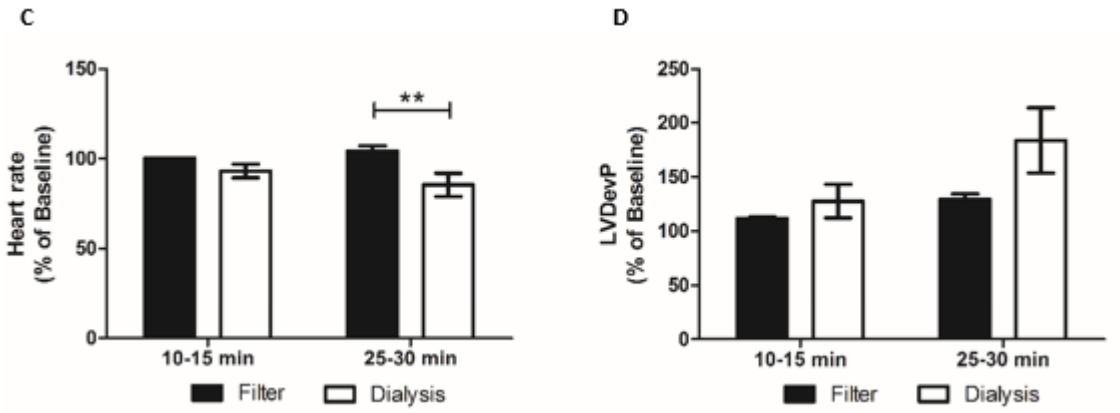

Figure 3.3: Characterization and comparison of the filter and dialysis method in lean male mice. (C) Heart rate (D) LVDevP (n=6). Significance is expressed as **p<0.01 versus dialysis 25-30 min. LVDevP: left ventricular developed pressure, min: minutes.

Thus these data demonstrate the filtering method to be equally useful for fatty acid conjugations and hence was employed for all the perfusion experiments completed in this study.

	Filter versus dialysis method (start of experiment)			**Filter versus dialysis method (end of experiment)**			**Time course**	
Parameters	Filter 10-15 min	Dialysis 10-15 min	Filter vs. Dialysis: 10-15 min	Filter 25-30 min	Dialysis 25-30 min	Filter vs. Dialysis: 25-30 min	Filter Start to End	Dialysis Start to End
RPP (%)	112.23 ± 1.15	116.32 ± 8.47	ns	134.38 ± 2.84	147.72 ± 11.63	ns	ns	$
$(dp/dt)_{max}$ (%)	111.08 ± 1.74	125.92 ± 13.78	ns	125.55 ± 6.32	164.65 ± 16.68	*	ns	$
Heart rate (%)	100.26 ± 0.22	93.17 ± 3.80	ns	104.32 ± 2.85	85.43 ± 6.52	**	ns	ns
LVDevP (%)	111.94 ± 1.03	127.69 ± 15.48	ns	129.50 ± 5.44	184.04 ± 30.18	ns	ns	ns
LVEDP (%)	98.87 ± 0.20	98.83 ± 0.53	ns	95.59 ± 0.58	97.36 ± 0.48	*	###	ns
LVESP (%)	100.21 ± 0.25	100.75 ± 0.40	ns	99.33 ± 0.78	101.21 ± 0.47	ns	ns	ns

Table 3.1: Characterization and comparison of the filter and dialysis method in lean male mice. Data are expressed as a percentage of baseline (n=6). Significance is expressed as *p<0.05; **p<0.01 versus dialysis 25-30min, ###p<0.001 versus filter start and $p<0.05 versus dialysis start. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, min: minutes, ns: not significant.

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3.3 Establishing the *ex vivo* mouse model of acute heart failure

The rapid switch in our perfusion system from the baseline/stabilization phase to the CAHF phase (reflecting *de novo* AHF) resulted in a drastic decrease in both RPP ($p<0.001$) and $(dp/dt)_{max}$ ($p<0.001$) (Figure 3.4). Switching from the CAHF to the RAHF phase (reflecting partial recovery) resulted in modest increases for RPP ($p<0.001$) and $(dp/dt)_{max}$ ($p<0.01$) (Figure 3.4, Table 3.2). However, the resultant recovery in the RAHF phase was still statistically lower than the initial baseline for both RPP ($p<0.001$) and $(dp/dt)_{max}$ ($p<0.001$).

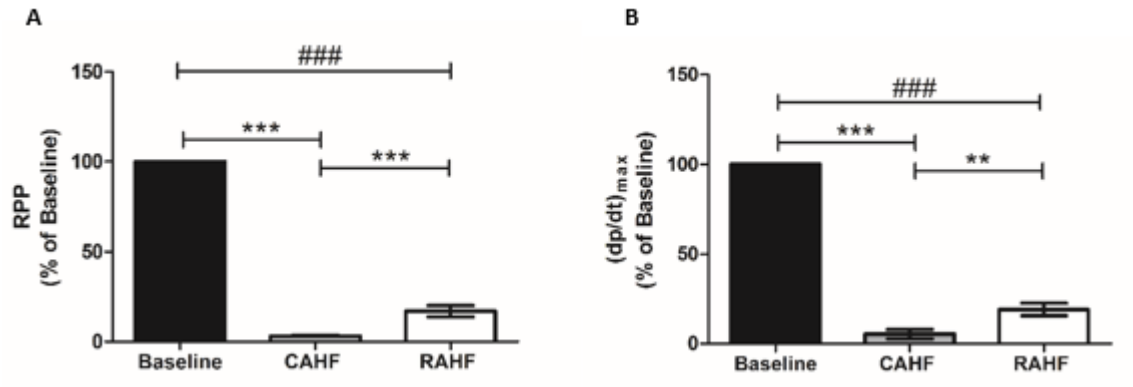

Figure 3.4: RPP and $(dp/dt)_{max}$ in establishing the *ex vivo* mouse model of acute heart failure (AHF) in lean male mice. Data is expressed as a percentage of baseline (n=6). Data represents the last ten minutes of each phase. Significance is expressed as **p<0.01; ***p<0.001 versus CAHF and ###p<0.001 versus RAHF. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure, RAHF: recovery acute heart failure.

There were no significant changes in heart rate between the phases, although there was a definite tendency for higher heart rates in the CAHF phase compared to the baseline. Heart rate during the RAHF phase was also slightly elevated compared to baseline (not significant), reflecting that the heart has sustained some damage even though there is some recovery (Table 3.2).

LVEDP decreased following the switch from baseline to CAHF ($p<0.01$) and subsequently increased during the RAHF phase compared to CAHF ($p<0.001$). There was a tendency for the LVEDP to be higher during RAHF compared to baseline, although this was not significant ($p>0.05$). The LVESP followed a similar pattern, although it was significantly lower during RAHF versus the initial baseline ($p<0.05$) (Table 3.2).

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Parameters	Baseline vs. CAHF	CAHF Phase (% of Baseline)	CAHF vs. RAHF	RAHF Phase (% of Baseline)	Baseline vs. RAHF
RPP (%)	***	3.12 ± 0.62	***	16.94 ± 3.08	###
$(dp/dt)_{max}$ (%)	***	2.97 ± 0.88	**	19.12 ± 3.43	###
Heart rate (%)	ns	274.70 ± 81.42	ns	118.90 ± 12.72	ns
LVDevP (%)	***	6.40 ± 4.12	ns	15.23 ± 3.31	###
LVEDP (%)	**	65.06 ± 6.89	***	120.30 ± 6.60	ns
LVESP (%)	***	46.47 ± 5.75	***	86.11 ± 1.61	#

Table 3.2: Establishing the *ex vivo* mouse model of acute heart failure (AHF) in lean male mice. Data from the CAHF and RAHF phases were normalised to baseline (assume 100%) and therefore CAHF and RAHF phase data are expressed as a percentage of baseline (n=6)**.** Data represents the last ten minutes of each phase. Significance is expressed as **p<0.01; ***p<0.001 versus CAHF and #p<0.05; ###p<0.001 versus RAHF. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, RAHF: recovery acute heart failure, ns: not significant.

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3.4 Effects of trimetazidine treatment with acute heart failure

Results will be discussed by first focusing on heart function during the CAHF phase, thereafter data for the RAHF phase will be discussed. It is important to note that the CAHF phase perfusion data are expressed as CAHF/Baseline (%), (using the RAW values obtained, see Addendum E) reflecting by what percentage the heart's function changes in relation to baseline. Similarly, the RAHF phase data are expressed as RAHF/CAHF (%), i.e. the "recovery" of heart function from the CAHF phase.

3.4.1 Acute heart failure: males

3.4.1.1 CAHF phase

The model results in a profound decline in RPP and $(dp/dt)_{max}$ in control lean males, i.e. to ~3.1% and 3.0%, respectively (Figure 3.5). Lean males treated with TMZ during the CAHF phase showed no significant changes for RPP, $(dp/dt)_{max}$ or any of the other parameters assessed (Figure 3.5, Table 3.3). Control obese males also displayed a robust decrease in RPP and $(dp/dt)_{max}$. Obese males showed a significant, yet negligible increase for RPP with TMZ treatment during the CAHF phase. However, none of the other parameters showed significant changes, except for an increase in LVDevP, a reflection of the increased RPP (Figure 3.5, Table 3.4).

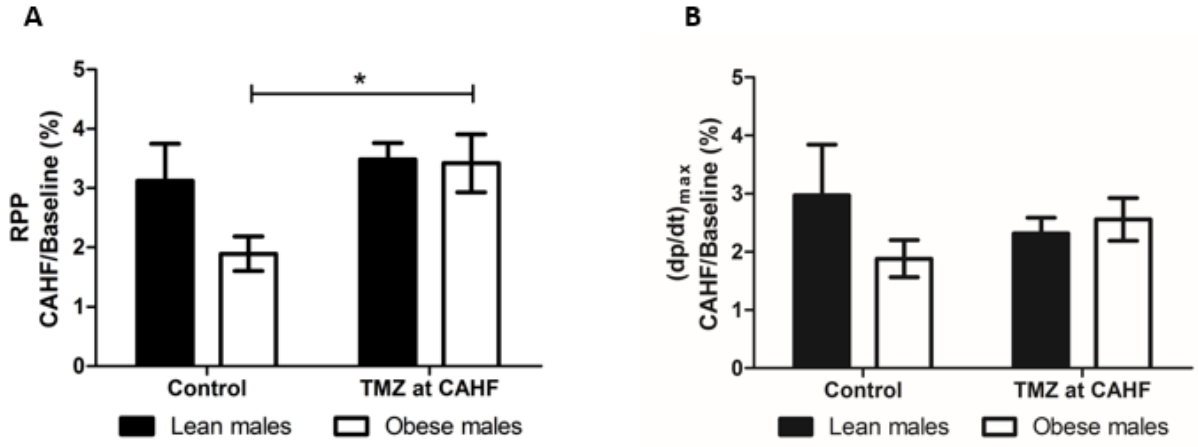

Figure 3.5 Effect of trimetazidine (TMZ) treatment during the CAHF phase on RPP and $(dp/dt)_{max}$ of lean and obese males. (A) RPP (B) $(dp/dt)_{max}$ (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. Significance is expressed as *p<0.05 versus control obese males. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure.

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TMZ treatment of lean males during CAHF						
Treatment group	**RPP (%)**	**Sig.**	**$(dp/dt)_{max}$ (%)**	**Sig.**	**LVDevP (%)**	**Sig.**
Control	3.12 ± 0.63	ns	2.97 ± 0.88	ns	6.40 ± 4.12	ns
TMZ at CAHF	3.48 ± 0.28		2.32 ± 0.26		0.87 ± 0.09	
Treatment group	**Heart rate (%)**	**Sig.**	**LVEDP (%)**	**Sig.**	**LVESP (%)**	**Sig.**
Control	274.74 ± 81.42	ns	65.06 ± 6.89	ns	46.47 ± 5.75	ns
TMZ at CAHF	407.79 ± 16.88		82.65 ± 4.68		55.40 ± 3.30	

Table 3.3 Effect of trimetazidine (TMZ) treatment during the CAHF phase (lean males) (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, Sig.: significance, ns: not significant.

TMZ treatment of obese males during CAHF						
Treatment Group	**RPP (%)**	**Sig.**	**$(dp/dt)_{max}$ (%)**	**Sig.**	**LVDevP (%)**	**Sig.**
Control	1.89 ± 0.29	*	1.89 ± 0.32	ns	0.49 ± 0.07	*
TMZ at CAHF	3.42 ± 0.49		2.56 ± 0.37		0.86 ± 0.12	
Treatment group	**Heart rate (%)**	**Sig.**	**LVEDP (%)**	**Sig.**	**LVESP (%)**	**Sig.**
Control	382.43 ± 11.68	ns	88.31 ± 3.07	ns	62.29 ± 5.08	ns
TMZ at CAHF	401.55 ± 22.64		76.20 ± 5.19		56.83 ± 4.85	

Table 3.4 Effect of trimetazidine (TMZ) treatment during the CAHF phase (obese males) (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. Significance is expressed as *$p<0.05$ versus control. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure Sig.: significance, ns: not significant.

There were no significant differences when comparing lean and obese males for the CAHF phase, although there was a tendency for the control obese males to have lowered RPP and $(dp/dt)_{max}$. However, control obese males displayed a higher LVEDP ($p<0.05$). TMZ treatment during the CAHF phase eliminated differences in LVEDP that were evident in control males (Figure 3.5, Table 3.5).

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Comparing TMZ treatment for lean vs. obese males during CAHF						
	Control			**TMZ during CAHF**		
Parameters	**Lean**	**Obese**	**Sig.**	**Lean**	**Obese**	**Sig.**
RPP (%)	3.12 ± 0.63	1.89 ± 0.29	ns	3.48 ± 0.28	3.42 ± 0.49	ns
$(dp/dt)_{max}$ (%)	2.97 ± 0.88	1.89 ± 0.32	ns	2.32 ± 0.26	2.56 ± 0.37	ns
Heart rate (%)	274.74 ± 81.42	382.43 ± 11.68	ns	407.79 ± 16.88	401.55 ±2 2.64	ns
LVDevP (%)	6.40 ± 4.12	0.49 ± 0.07	ns	0.87 ± 0.09	0.86 ± 0.12	ns
LVEDP (%)	65.06 ± 6.89	88.31 ± 3.07	*	82.65 ± 4.68	76.20 ± 5.19	ns
LVESP (%)	46.47 ± 5.75	62.29 ± 5.08	ns	55.40 ± 3.30	56.83 ± 4.85	ns

Table 3.5 Comparing the effect of trimetazidine (TMZ) treatment during the CAHF phase (lean and obese males) (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. Significance is expressed as *p<0.05 versus lean males. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, Sig.: significance, ns: not significant.

3.4.1.2 RAHF phase

The control lean males showed a respective 5.6-fold and 6.4-fold increase for RPP and $(dp/dt)_{max}$ from the previous CAHF phase. TMZ treatment initiated during the CAHF phase did not make any difference in terms of functionality. However, when TMZ treatment was initiated during the RAHF phase, this strongly improved RPP and $(dp/dt)_{max}$ values compared to both control and the CAHF treated lean males. Here data revealed an impressive 3.2-fold increase in RPP between RAHF-treated and control lean males ($p<0.001$) and a similar 2.9-fold increase ($p<0.001$) versus CAHF-treated lean males. The other significant parameters that increased included $(dp/dt)_{max}$ and LVDevP (Figure 3.6, Table 3.6).

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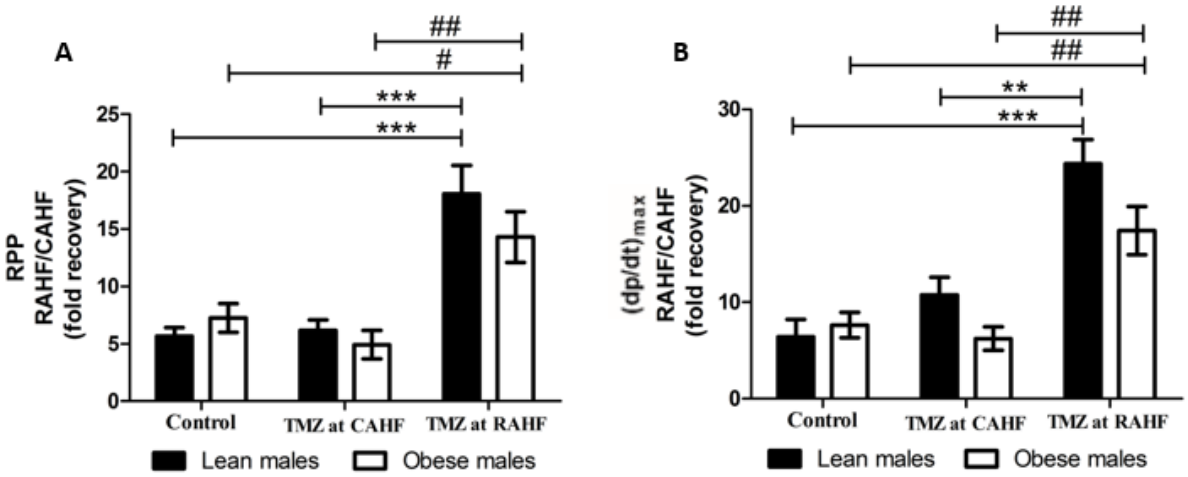

Figure 3.6 Effect of trimetazidine (TMZ) treatment during the RAHF phase on RPP and $(dp/dt)_{max}$ of lean and obese males. (A) RPP (B) $(dp/dt)_{max}$ (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a fold recovery from the previous CAHF phase. Significance is expressed as **p<0.01; ***p<0.001 versus TMZ at RAHF lean males and #p<0.05; ##p<0.01 versus TMZ at RAHF obese males. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure, RAHF: recovery acute heart failure.

The obese males exhibited a similar pattern as for the lean males and recovered similarly during the RAHF phase. Moreover, TMZ treatment initiated during the RAHF phase also significantly improved RPP and $(dp/dt)_{max}$ for the obese males (Figure 3.6, Table 3.7).

When performing comparative analysis for the RAHF phase, there was a tendency for TMZ-treated lean males to achieve higher performance results, although this was not statistically significant (Table 3.8).

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TMZ treatment of lean males during RAHF						
Treatment group	**RPP (%)**	**Sig.**	**$(dp/dt)_{max}$ (%)**	**Sig.**	**LVDevP (%)**	**Sig.**
Control	568.43 ± 71.91	***	640.47 ± 184.14	***	1259.06 ± 485.90	***
TMZ at CAHF	616.08 ± 91.27	***	1077.15 ± 184.60	**	2204.29 ± 355.33	***
TMZ at RAHF	1806.94 ± 245.55		2435.16 ± 253.84		6025.70 ± 654.65	
Treatment group	**Heart rate (%)**	**Sig.**	**LVEDP (%)**	**Sig.**	**LVESP (%)**	**Sig.**
Control	210.13 ± 127.11	ns	201.85 ± 36.57	ns	211.06 ± 43.49	ns
TMZ at CAHF	28.26 ± 1.48		160.23 ± 9.38		170.36 ± 11.07	
TMZ at RAHF	29.74 ± 2.23		158.26 ± 18.72		171.80 ± 24.07	

Table 3.6 Effect of trimetazidine (TMZ) treatment during the RAHF phase (lean males) (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a percentage of CAHF. Significance is expressed as **p<0.01; ***p<0.001 versus TMZ at RAHF. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, RAHF: recovery acute heart failure, Sig.: significance, ns: not significant.

TMZ treatment of obese males during RAHF						
Treatment group	**RPP (%)**	**Sig.**	**$(dp/dt)_{max}$ (%)**	**Sig.**	**LVDevP (%)**	**Sig.**
Control	724.78 ± 124.26	*	763.84 ± 132.18	**	2411.10 ± 458.16	**
TMZ at CAHF	492.12 ± 122.78	**	624.06 ± 122.29	**	1678.49 ± 470.27	
TMZ at RAHF	1430.89 ± 221.17		1744.19 ± 249.20		4522.35 ± 742.07	
Treatment group	**Heart rate (%)**	**Sig.**	**LVEDP (%)**	**Sig.**	**LVESP (%)**	**Sig.**
Control	31.75 ± 2.85	ns	142.48 ± 10.43	ns	147.98 ± 11.77	ns
TMZ at CAHF	30.17 ± 2.62		158.98 ± 15.10		164.74 ± 16.48	
TMZ at RAHF	31.78 ± 2.38		141.43 ± 9.98		147.96 ± 12.18	

Table 3.7 Effect of trimetazidine (TMZ) treatment during the RAHF phase (obese males) (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a percentage of CAHF. Significance is expressed as *p<0.05; **p<0.01 versus TMZ at RAHF. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, RAHF: recovery acute heart failure, Sig.: significance, ns: not significant.

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Comparing TMZ treatment of lean vs. obese males during RAHF						
	Control					
	Lean			**Obese**		**Sig.**
RPP (%)	568.43 ± 71.91			724.78 ± 124.26		ns
$(dp/dt)_{max}$ (%)	640.47 ± 184.14			763.84 ± 132.18		ns
Heart rate (%)	210.13 ± 127.11			31.75 ± 2.85		ns
LVDevP (%)	1259.06 ± 485.90			2411.10 ± 458.16		ns
LVEDP (%)	201.85 ± 36.57			142.48 ± 10.43		ns
LVESP (%)	211.06 ± 43.49			147.98 ± 11.77		ns
	TMZ at CAHF			**TMZ at RAHF**		
	Lean	**Obese**	**Sig.**	**Lean**	**Obese**	**Sig.**
RPP (%)	616.08 ± 91.27	492.12 ± 122.78	ns	1806.94 ± 245.55	1430.89 ± 221.17	ns
$(dp/dt)_{max}$ (%)	1077.15 ± 184.60	624.06 ± 122.29	ns	2435.16 ± 253.84	1744.19 ± 249.20	ns
Heart rate (%)	28.26 ± 1.48	30.17 ± 2.62	ns	29.74 ± 2.23	31.78 ± 2.38	ns
LVDevP (%)	2204.29 ± 355.33	1678.49 ± 470.27	ns	6025.70 ± 654.65	4522.35 ± 742.07	ns
LVEDP (%)	160.23 ± 9.38	158.98 ± 15.10	ns	158.26 ± 18.72	141.43 ± 9.98	ns
LVESP (%)	170.36 ± 11.07	164.74 ± 16.48	ns	171.80 ± 24.07	147.96 ± 12.18	ns

Table 3.8 Comparing the effect of trimetazidine (TMZ) treatment during the RAHF phase (lean and obese males) (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a percentage of CAHF. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, RAHF: recovery acute heart failure Sig.: significance, ns: not significant.

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3.4.1.3 Summary

The *ex vivo* AHF model results in a profound decrease in heart function during the CAHF phase that is moderately improved during the RAHF phase. Generally, TMZ treatment during the CAHF phase did not result in significant improvement for lean and obese hearts although there was a small but significant increase in RPP for obese males. During the RAHF phase lean and obese hearts improve without treatment. However, TMZ treatment during this phase resulted in a robust increase in function for both lean and obese males.

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3.4.2 Acute heart failure: females

Females were divided into the two main stages of the estrus cycle, namely the follicular and luteal phase. However, obese females experienced stasis in the luteal phase. Thus we initially focused on differences between the phases of the lean animals followed by a comparison of lean and obese females (luteal phases).

3.4.2.1 Lean follicular vs. lean luteal

3.4.2.1.1 CAHF phase

Control lean follicular females displayed significantly lower RPP and $(dp/dt)_{max}$ versus the initial baseline. Treatment of lean follicular females with TMZ in the CAHF phase resulted in a non-significant decrease in RPP, although the detrimental decrease in $(dp/dt)_{max}$ was significant ($p<0.05$) (Figure 3.7, Table 3.9). Of note, the relative degree of these changes (even though significant) are very minimal. No changes were found for any of the other parameters (Table 3.9).

Control lean luteal females showed similar patterns for RPP and $(dp/dt)_{max}$ with and without TMZ treatment initiated during the CAHF phase (Figure 3.7, Table 3.10).

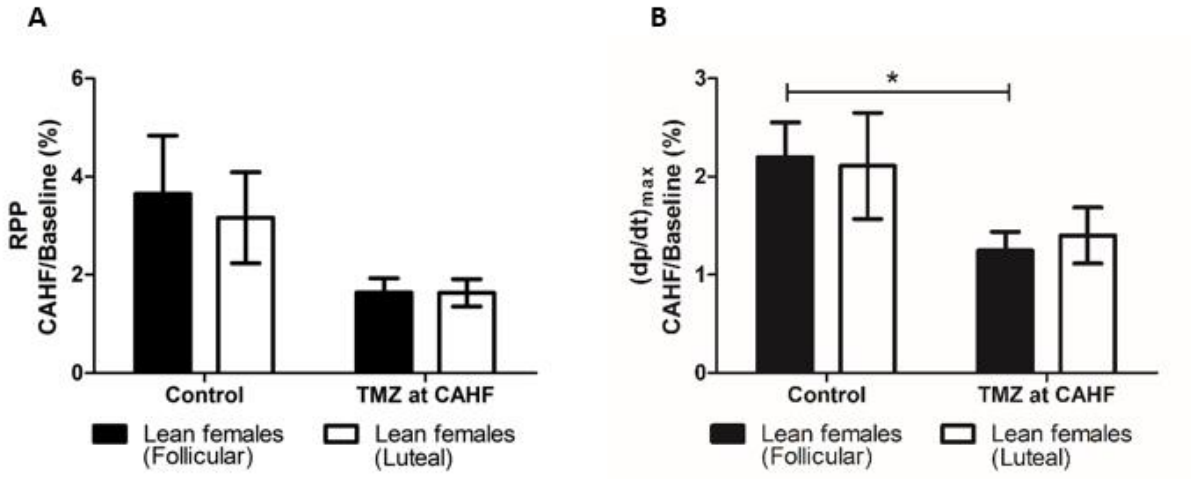

Figure 3.7: Effect of trimetazidine (TMZ) treatment during the CAHF phase on RPP and $(dp/dt)_{max}$ of lean females (follicular and luteal phase). (A) RPP (B) $(dp/dt)_{max}$ (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. Significance is expressed as *$p<0.05$ versus control follicular females. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure.

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TMZ treatment of lean females (follicular) during CAHF						
Treatment group	**RPP (%)**	**Sig.**	**$(dp/dt)_{max}$ (%)**	**Sig.**	**LVDevP (%)**	**Sig.**
Control	3.65 ± 1.18	ns	2.20 ± 0.36	*	1.42 ± 0.53	ns
TMZ at CAHF	1.64 ± 0.29		1.25 ± 0.19		0.47 ± 0.14	
Treatment group	**Heart rate (%)**	**Sig.**	**LVEDP (%)**	**Sig.**	**LVESP (%)**	**Sig.**
Control	370.14 ± 42.40	ns	79.33 ± 4.02	ns	63.26 ± 4.62	ns
TMZ at CAHF	390.47 ± 34.75		88.56 ± 9.22		69.06 ± 8.36	

Table 3.9: Effect of trimetazidine (TMZ) treatment during the CAHF phase (lean follicular females) (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. Significance is expressed as *p<0.05 versus control follicular females. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, Sig.: significance, ns: not significant.

TMZ treatment of lean females (luteal) during CAHF						
Treatment group	**RPP (%)**	**Sig.**	**$(dp/dt)_{max}$ (%)**	**Sig.**	**LVDevP (%)**	**Sig.**
Control	3.17 ± 0.93	ns	2.11 ± 0.54	ns	1.26 ± 0.57	ns
TMZ at CAHF	1.63 ± 0.27		1.40 ± 0.29		0.47 ± 0.06	
Treatment group	**Heart rate (%)**	**Sig.**	**LVEDP (%)**	**Sig.**	**LVESP (%)**	**Sig.**
Control	326.97 ± 36.00	ns	65.49 ± 4.01	ns	46.76 ± 3.93	ns
TMZ at CAHF	347.26 ± 30.05		78.67 ± 9.69		57.91 ± 8.55	

Table 3.10: Effect of trimetazidine (TMZ) treatment during the CAHF phase (lean luteal females) (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, Sig.: significance, ns: not significant.

When comparing lean follicular and lean luteal control females in the CAHF phase, no differences were observed for the functional parameters RPP and $(dp/dt)_{max}$. However, differences were found for LVEDP and LVESP. Here lean follicular control females exhibited a moderately higher LVEDP ($p<0.05$) and LVESP ($p<0.05$). TMZ-treated lean females showed similar responses, although differences in the LVESP and LVEDP were no longer evident (Figure 3.7, Table 3.11).

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Comparing TMZ treatment for lean follicular vs. lean luteal females during CAHF						
	Control			TMZ at CAHF		
Parameters	**Follicular**	**Luteal**	**Sig.**	**Follicular**	**Luteal**	**Sig.**
RPP (%)	3.65 ± 1.18	3.17 ± 0.93	ns	1.64 ± 0.29	1.63 ± 0.27	ns
$(dp/dt)_{max}$ (%)	2.20 ± 0.36	2.11 ± 0.54	ns	1.25 ± 0.19	1.40 ± 0.29	ns
Heart rate (%)	370.14 ± 42.40	326.97 ± 36.00	ns	390.47 ± 34.75	347.26 ± 30.05	ns
LVDevP (%)	1.42 ± 0.53	1.26 ± 0.57	ns	0.47 ± 0.14	0.47 ± 0.06	ns
LVEDP (%)	79.33 ± 4.02	65.49 ± 4.01	*	88.56 ± 9.22	78.67 ± 9.69	ns
LVESP (%)	63.26 ± 4.62	46.76 ± 3.93	*	69.06 ± 8.36	57.91 ± 8.55	ns

Table 3.11: Comparing the effect of trimetazidine (TMZ) treatment during the CAHF phase (lean follicular and luteal females) (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. Significance is expressed as *p<0.05 versus lean follicular females. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, Sig.: significance, ns: not significant.

3.4.2.1.2 RAHF phase

Control lean follicular females showed a respective 8.4-fold and 16.2-fold increase in RPP and $(dp/dt)_{max}$ from the previous CAHF phase (Figure 3.8). We noted a delayed treatment effect of TMZ in lean follicular females during the RAHF phase. Here we previously found that treatment initiated during the CAHF phase negatively affected functional parameters when measured at CAHF phase (Figure 3.7). However, the same females now display a robust increase in RPP ($p<0.05$) that is 2.4-fold higher (and LVDevP 2.8 fold higher) compared to control females when assessed during the RAHF phase. Analysis of $(dp/dt)_{max}$ did not mirror the significance, although it followed the same pattern as for RPP (Figure 3.8, Table 3.12).

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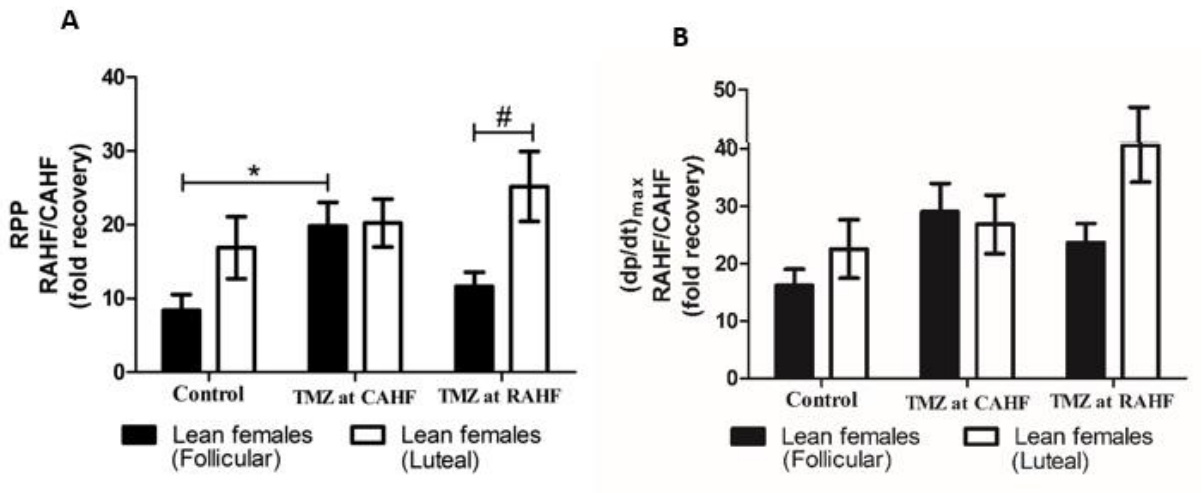

Figure 3.8: Effect of trimetazidine (TMZ) treatment during the RAHF phase on RPP and $(dp/dt)_{max}$ of lean females (follicular and luteal phase). (A) RPP (B) $(dp/dt)_{max}$ (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a fold recovery from the previous CAHF phase. Significance is expressed as *p<0.05 versus control lean follicular females and #p<0.05 versus TMZ at RAHF lean follicular females. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure, RAHF: recovery acute heart failure.

Control lean luteal females showed a substantial 16.9-fold and 22.6-fold increase in RPP and $(dp/dt)_{max}$, respectively, from the previous CAHF phase. TMZ treatment of lean luteal females during the RAHF phase did not result in any significant changes although there was a tendency to respond in a graded fashion over the different treatment groups (Figure 3.8, Table 3.13).

When comparing follicular and luteal lean females, there were no significant differences except for TMZ treatment in the RAHF phase where lean luteal females displayed a high RPP ($p<0.05$) (Figure 3.8, Table 3.14).

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TMZ treatment of lean females (follicular) during RAHF						
Treatment group	**RPP (%)**	**Sig.**	**$(dp/dt)_{max}$ (%)**	**Sig.**	**LVDevP (%)**	**Sig.**
Control	839.55 ± 213.56	*	1624.63 ± 283.02	ns	2875.81 ± 725.74	*
TMZ at CAHF	1980.56 ± 321.98		2911.24 ± 487.59		8049.63 ± 1961.21	
TMZ at RAHF	1163.93 ± 191.79		2364.91 ± 338.16		4918.75 ± 762.73	
Treatment group	**Heart rate (%)**	**Sig.**	**LVEDP (%)**	**Sig.**	**LVESP (%)**	**Sig.**
Control	30.21 ± 4.14	ns	140.87 ± 9.40	ns	149.10 ± 10.88	ns
TMZ at CAHF	27.94 ± 3.51		139.35 ± 24.49		152.26 ± 30.49	
TMZ at RAHF	23.93 ± 2.00		137.35 ± 3.71		145.29 ± 4.73	

Table 3.12: Effect of trimetazidine (TMZ) treatment during the RAHF phase (lean follicular females) (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a percentage of CAHF. Significance is expressed as *p<0.05 versus control. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, RAHF: recovery acute heart failure, Sig.: significance, ns: not significant.

TMZ treatment of lean females (luteal) during RAHF						
Treatment group	**RPP (%)**	**Sig.**	**$(dp/dt)_{max}$ (%)**	**Sig.**	**LVDevP (%)**	**Sig.**
Control	1687.62 ± 421.02	ns	2255.71 ± 507.28	ns	6116.62 ± 1675.88	ns
TMZ at CAHF	2021.86 ± 324.85		2685.27 ± 513.67		6202.00 ± 1064.64	
TMZ at RAHF	2519.13 ± 475.16		4060.05 ± 639.68		9475.80 ± 1807.90	
Treatment group	**Heart rate (%)**	**Sig.**	**LVEDP (%)**	**Sig.**	**LVESP (%)**	**Sig.**
Control	33.55 ± 4.86	ns	179.64 ± 10.82	ns	199.59 ± 13.44	ns
TMZ at CAHF	34.18 ± 5.60		176.24 ± 43.51		194.08 ± 51.29	
TMZ at RAHF	27.31 ± 1.41		134.06 ± 4.27		147.00 ± 6.88	

Table 3.13: Effect of trimetazidine (TMZ) treatment during the RAHF phase (lean luteal females) (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a percentage of CAHF. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, RAHF: recovery acute heart failure, Sig.: significance, ns: not significant.

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Comparing TMZ treatment for lean follicular vs. lean luteal females during RAHF						
	Control					
Parameters	**Follicular**			**Luteal**		**Sig.**
RPP (%)	839.55 ± 213.56			1687.62 ± 421.02		ns
$(dp/dt)_{max}$ (%)	1624.63 ± 283.02			2255.71 ± 507.28		ns
Heart rate (%)	30.21 ± 4.14			33.55 ± 4.86		ns
LVDevP (%)	2875.81 ± 725.74			6116.62 ± 1675.88		ns
LVEDP (%)	140.87 ± 9.40			179.64 ± 10.82		ns
LVESP (%)	149.10 ± 10.88			199.59 ± 13.44		ns
	TMZ at CAHF			**TMZ at RAHF**		
	Follicular	**Luteal**	**Sig.**	**Follicular**	**Luteal**	**Sig.**
RPP (%)	1980.56 ± 321.98	2021.86 ± 324.85	ns	1163.93 ± 191.79	2519.13 ± 475.16	*
$(dp/dt)_{max}$ (%)	2911.24 ± 487.59	2685.27 ± 513.67	ns	2364.91 ± 338.16	4060.05 ± 639.68	ns
Heart rate (%)	27.94 ± 3.51	34.18 ± 5.60	ns	23.93 ± 2.00	27.31 ± 1.41	ns
LVDevP (%)	8049.63 ± 1961.21	6202.00 ± 1064.64	ns	4918.75 ± 762.73	9475.80 ± 1807.90	ns
LVEDP (%)	139.35 ± 24.49	176.24 ± 43.51	ns	137.35 ± 3.71	134.06 ± 4.27	ns
LVESP (%)	152.26 ± 30.49	194.08 ± 51.29	ns	145.29 ± 4.73	147.00 ± 6.88	ns

Table 3.14: Comparing the effect of trimetazidine (TMZ) treatment during the RAHF phase (lean follicular and luteal females) (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a percentage of CAHF. Significance is expressed as *p<0.05 versus follicular females. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, RAHF: recovery acute heart failure, Sig.: significance, ns: not significant.

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3.4.2.1.3 Summary

The data reveal that during the CAHF phase lean females (follicular and luteal) show similar decreases in functionality. Treatment with TMZ during CAHF results in early detrimental effects, although this shows a delayed positive effect later-on during the RAHF phase (lean follicular females). During the RAHF phase, lean luteal females show superior recovery from the previous CAHF phase versus follicular females. Although TMZ treatment show no significant effect for lean luteal females, RAHF-treated luteal females show superior functioning compared to follicular females. Thus it seems that luteal females possess an inherent ability to better cope with an AHF event and that TMZ treatment does not contribute to this. However, lean follicular females benefit from TMZ treatment during the CAHF phase.

3.4.2.2 Lean luteal vs. obese luteal

3.4.2.2.1 CAHF phase

Control obese luteal females exhibited a similar decrease to lean females for RPP and $(dp/dt)_{max}$ during the CAHF phase. TMZ treatment of obese luteal females showed the same pattern as for lean females with a decrease for both RPP ($p<0.01$) and $(dp/dt)_{max}$ ($p<0.05$) (Figure 3.9). TMZ treatment also resulted in a considerable increase in LVEDP ($p<0.01$) and LVESP ($p<0.01$) for obese luteal females (Figure 3.9, Table 3.15).

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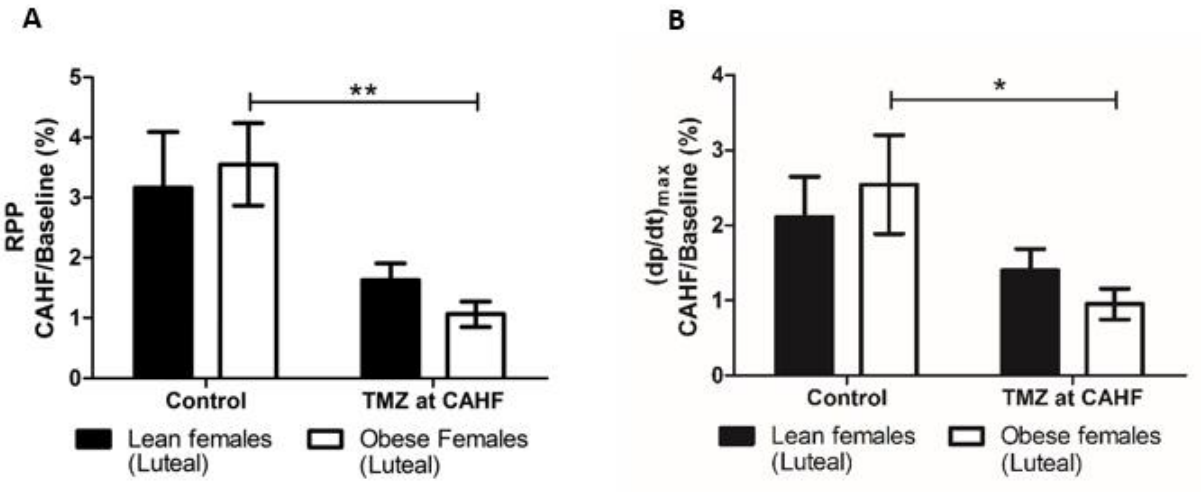

Figure 3.9: Effect of trimetazidine (TMZ) treatment during the CAHF phase on RPP and $(dp/dt)_{max}$ (lean and obese females). (A) RPP (B) $(dp/dt)_{max}$ (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure.

For the CAHF phase, the only statistical difference between obese and lean luteal females was a high LVESP ($p<0.05$) found in lean control luteal females. TMZ increased the LVESP of obese females, thereby eliminating the difference with control animals (Table 3.16).

TMZ treatment of obese females (luteal) during CAHF						
Treatment group	**RPP (%)**	**Sig.**	**$(dp/dt)_{max}$ (%)**	**Sig.**	**LVDevP (%)**	**Sig.**
Control	3.55 ± 0.69	**	2.54 ± 0.66	*	1.40 ± 0.55	ns
TMZ at CAHF	1.07 ± 0.21		0.95 ± 0.21		0.32 ± 0.05	
Treatment group	**Heart rate (%)**	**Sig.**	**LVEDP (%)**	**Sig.**	**LVESP (%)**	**Sig.**
Control	339.53 ± 48.09	ns	47.29 ± 10.60	**	28.88 ± 6.73	**
TMZ at CAHF	335.46 ± 34.69		87.69 ± 5.43		62.80 ± 4.86	

Table 3.15: Effect of trimetazidine (TMZ) treatment during the CAHF phase (obese luteal females) (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. Significance is expressed as *$p<0.05$; **$p<0.01$ versus control. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, Sig.: significance, ns: not significant.

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Comparing TMZ treatment for lean vs. obese (luteal) females during CAHF						
	Control			TMZ at CAHF		
Parameters	**Lean**	**Obese**	**Sig.**	**Lean**	**Obese**	**Sig.**
RPP (%)	3.17 ± 0.93	3.55 ± 0.69	ns	1.63 ± 0.27	1.07 ± 0.21	ns
$(dp/dt)_{max}$ (%)	2.11 ± 0.54	2.54 ± 0.66	ns	1.40 ± 0.29	0.95 ± 0.21	ns
Heart rate (%)	326.97 ± 36.00	339.53 ± 48.09	ns	347.26 ± 30.05	335.46 ± 34.69	ns
LVDevP (%)	1.26 ± 0.57	1.40 ± 0.55	ns	0.47 ± 0.06	0.32 ± 0.05	ns
LVEDP (%)	65.49 ± 4.01	47.29 ± 10.60	ns	78.67 ± 9.69	87.69 ± 5.43	ns
LVESP (%)	46.76 ± 3.93	28.88 ± 6.73	*	57.91 ± 8.55	62.80 ± 4.86	ns

Table 3.16: Comparing the effect of trimetazidine (TMZ) treatment during the CAHF phase (lean and obese luteal females) (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. Significance is expressed as *p<0.05 versus lean females. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, Sig.: significance, ns: not significant.

3.4.2.2.2 RAHF phase

The control obese females showed a much lower recovery during the RAHF phase (Figure 3.10). However, obese females with TMZ treatment responded favorably with a 3.3-fold increase in RPP ($p<0.01$) and a 2.3-fold increase in $(dp/dt)_{max}$ ($p<0.05$) compared to control obese females (Figure 3.10, Table 3.17).

When comparing lean and obese luteal females during the RAHF phase it is evident that both groups respond in a graded fashion; however the lean luteal females consistently displayed a superior improvement in heart function. Here lean control females have a substantial advantage over the control obese females, e.g. RPP is 3.2-fold ($p<0.05$) higher versus obese females. Moreover, lean luteal females that receive TMZ treatment during the RAHF phase displayed a higher $(dp/dt)_{max}$ ($p<0.01$) compared to the obese females (Figure 3.9, Table 3.18).

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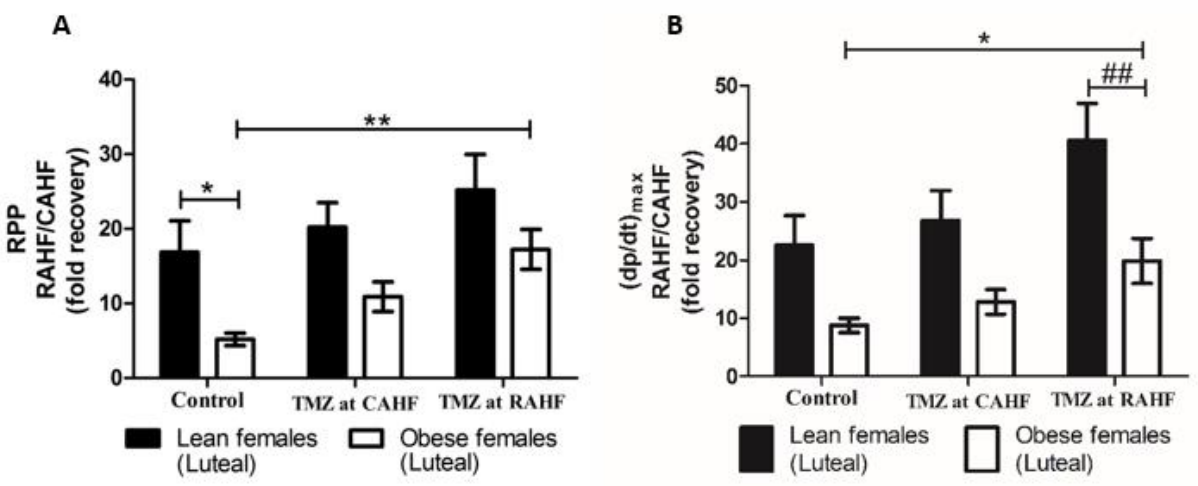

Figure 3.10: Effect of trimetazidine (TMZ) treatment during the RAHF phase on RPP and $(dp/dt)_{max}$ (lean and obese females). (A) RPP (B) $(dp/dt)_{max}$ (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a fold recovery from the previous CAHF phase. Significance is expressed as *p<0.05; **p<0.01 versus control obese females and ##p<0.01 versus TMZ at RAHF obese females. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure, RAHF: recovery acute heart failure.

TMZ treatment of obese females (luteal) during RAHF						
Treatment group	**RPP (%)**	**Sig.**	**$(dp/dt)_{max}$ (%)**	**Sig.**	**LVDevP (%)**	**Sig.**
Control	519.68 ± 83.40		876.00 ± 125.34		1725.24 ± 397.04	
TMZ at CAHF	1087.06 ± 199.76		1284.62 ± 211.00		3199.25 ± 743.72	
TMZ at RAHF	1722.53 ± 267.61	**	1984.13 ± 387.63	*	5400.37 ± 887.21	**
Treatment group	**Heart rate (%)**	**Sig.**	**LVEDP (%)**	**Sig.**	**LVESP (%)**	**Sig.**
Control	38.49 ± 10.56	ns	574.86 ± 308.07	ns	521.49 ± 240.36	ns
TMZ at CAHF	36.99 ± 3.84		140.93 ± 11.62		145.23 ± 12.91	
TMZ at RAHF	32.55 ± 2.48		126.83 ± 7.53		132.12 ± 9.01	

Table 3.17: Effect of trimetazidine (TMZ) treatment in the RAHF phase (obese luteal females) (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a percentage of CAHF. Significance is expressed as *p<0.05; **p<0.01 versus control. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, RAHF: recovery acute heart failure, Sig.: significance, ns: not significant.

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Comparing TMZ treatment of lean vs. obese (luteal) females during RAHF						
	Control					
Parameters	**Lean**			**Obese**		**Sig.**
RPP (%)	1687.62 ± 421.02			519.68 ± 83.40		*
$(dp/dt)_{max}$ (%)	2255.71 ± 507.28			876.00 ± 125.34		ns
Heart rate (%)	33.55 ± 4.86			38.49 ± 10.56		ns
LVDevP (%)	6116.62 ± 1675.88			1725.24 ± 397.04		*
LVEDP (%)	179.64 ± 10.82			574.86 ±308.07		ns
LVESP (%)	199.59 ± 13.44			521.49 ± 240.36		ns
	TMZ at CAHF			**TMZ at RAHF**		
	Lean	**Obese**	**Sig.**	**Lean**	**Obese**	**Sig.**
RPP (%)	2021.86 ± 324.85	1087.06 ± 199.76	ns	2519.13 ± 475.16	1722.53 ± 267.61	ns
$(dp/dt)_{max}$ (%)	2685.27 ± 513.67	1284.62 ± 211.00	ns	4060.05 ± 639.68	1984.13 ± 387.63	**
Heart rate (%)	34.18 ± 5.60	36.99 ± 3.84	ns	27.31 ± 1.41	32.55 ± 2.48	ns
LVDevP (%)	6202.00 ± 1064.64	3199.25 ± 743.72	ns	9475.80 ± 1807.90	5400.37 ± 887.21	ns
LVEDP (%)	176.24 ± 43.51	140.93 ± 11.62	ns	134.06 ± 4.27	126.83 ± 7.53	ns
LVESP (%)	194.08 ± 51.29	145.23 ± 12.91	ns	147.00 ± 6.88	132.12 ± 9.01	ns

Table 3.18: Comparing the effect of trimetazidine (TMZ) treatment during the RAHF phase (lean and obese luteal females) (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a percentage of CAHF. Significance is expressed as *p<0.05; **p<0.01 versus lean females. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, RAHF: recovery acute heart failure, Sig.: significance, ns: not significant.

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3.4.2.2.3 Summary

It is clear that the inherent ability of lean luteal females to handle an AHF event is lost with obesity. Here the lean control females displayed superior function and recovery from the previous CAHF phase and as such TMZ treatment has no further beneficial effects. Conversely, the control obese females start out with a limited recovery from CAHF, but with TMZ treatment during the RAHF phase this results in favorable effects although this is still not adequate to restore functionality to that of the lean females.

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3.4.3 Acute heart failure: males and females

3.4.3.1 Lean

3.4.3.1.1 CAHF phase

Lean control males and follicular females displayed a decrease in RPP and $(dp/dt)_{max}$ (Figure 3.11). The only difference is the relatively small increased LVESP in the females ($p<0.05$), although this is no longer the case with TMZ treatment during the CAHF phase. TMZ treated follicular females also have lowered RPP ($p<0.001$), $(dp/dt)_{max}$ ($p<0.01$) and LVDevP ($P<0.05$) compared to treated lean males, which is consistent considering the negative effect of TMZ on females treated during CAHF. (Figure 3.11, Table 3.19).

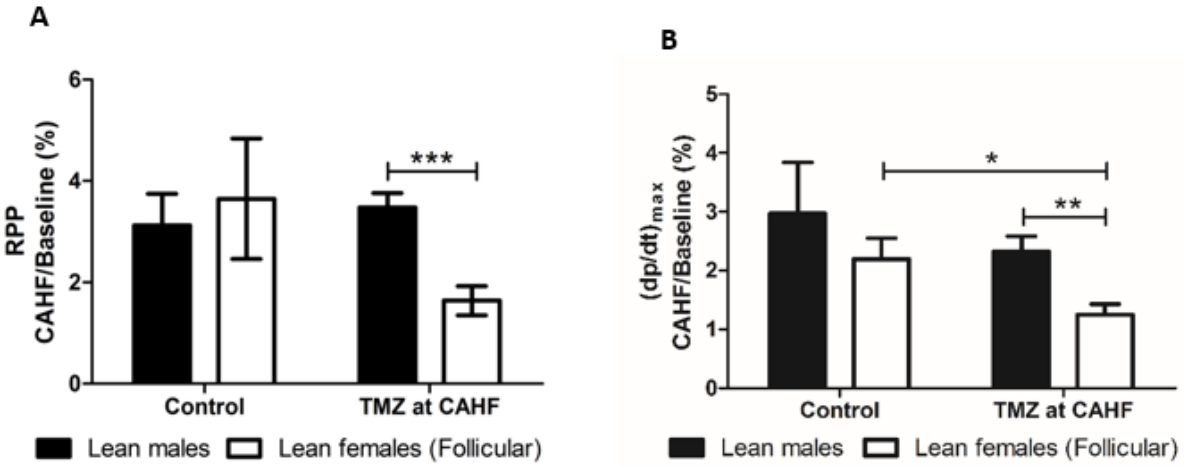

Figure 3.11: Effect of trimetazidine (TMZ) treatment during the CAHF phase on RPP and $(dp/dt)_{max}$ of lean males and lean follicular females. (A) RPP (B) $(dp/dt)_{max}$ (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. Significance is expressed as *p<0.05; **p<0.01; ***p<0.001 versus TMZ at CAHF lean follicular females. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure.

Lean control males and luteal females exhibited an attenuation in RPP and $(dp/dt)_{max}$ and there were no differences found for the CAHF phase. As for the follicular females, TMZ-treated luteal females displayed a slightly lower RPP ($p<0.001$) and $(dp/dt)_{max}$ ($p<0.05$) compared to the lean males (Figure 3.12, Table 3.20).

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Comparing TMZ treatment for lean males vs. lean follicular females during CAHF						
	Control			TMZ at CAHF		
Parameters	**Males**	**Females**	**Sig.**	**Males**	**Females**	**Sig.**
RPP (%)	3.12 ± 0.63	3.65 ± 1.18	ns	3.48 ± 0.80	1.64 ± 0.29	***
$(dp/dt)_{max}$ (%)	2.97 ± 0.88	2.20 ± 0.36	ns	2.32 ± 0.26	1.25 ± 0.19	**
Heart rate (%)	274.74 ± 81.42	370.14 ± 42.40	ns	407.79 ± 16.88	390.47 ± 34.75	ns
LVDevP (%)	6.40 ± 4.12	1.42 ± 0.53	ns	0.87 ± 0.09	0.47 ± 0.14	*
LVEDP (%)	65.06 ± 6.89	79.33 ± 4.02	ns	82.65 ± 4.68	88.55 ± 9.22	ns
LVESP (%)	46.47 ± 5.75	63.26 ± 4.62	*	55.40 ± 3.30	69.06 ± 8.36	ns

Table 3.19: Comparing the effect of trimetazidine (TMZ) treatment during the CAHF phase (lean males and lean follicular females) (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. Significance is expressed as *p<0.05; **p<0.01; ***p<0.001 versus lean males. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, Sig.: significance, ns: not significant.

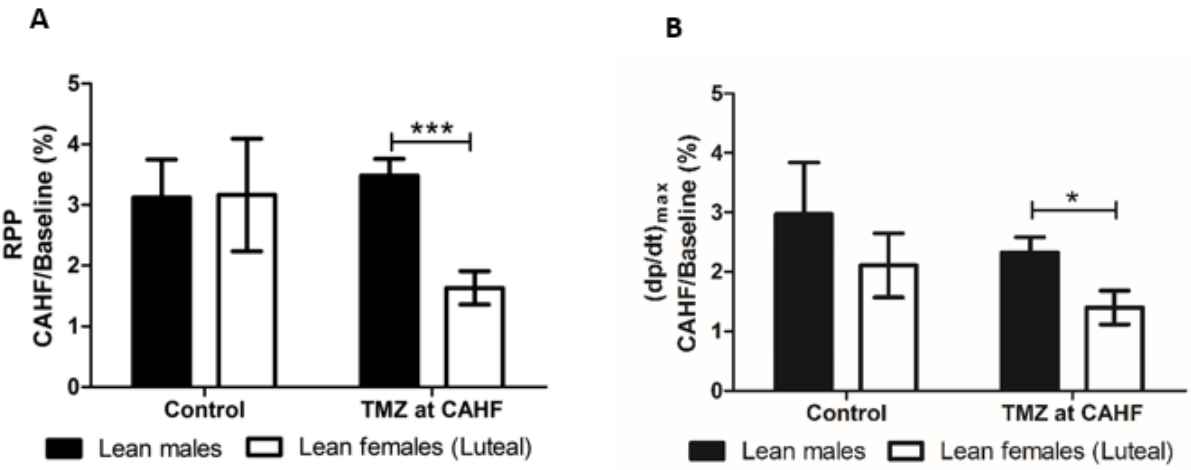

Figure 3.12: Effect of trimetazidine (TMZ) treatment during the CAHF phase on RPP and $(dp/dt)_{max}$ of lean males and lean luteal females. (A) RPP (B) $(dp/dt)_{max}$ (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. Significance is expressed as *p<0.05; ***p<0.001 versus TMZ at CAHF lean luteal females. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure.

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Comparing TMZ treatment for lean males vs. lean luteal females during CAHF						
	Control			TMZ at CAHF		
Parameters	**Males**	**Females**	**Sig.**	**Males**	**Females**	**Sig.**
RPP (%)	3.12 ± 0.63	3.17 ± 0.93	ns	3.48 ± 0.28	1.63 ± 0.27	***
$(dp/dt)_{max}$ (%)	2.97 ± 0.88	2.11 ± 0.54	ns	2.32 ± 0.26	1.40 ± 0.29	*
Heart rate (%)	274.74 ± 81.42	326.97 ± 36.00	ns	407.79 ± 16.88	347.26 ± 30.05	ns
LVDevP (%)	6.40 ± 4.12	1.26 ± 0.57	ns	0.87 ± 0.09	0.47 ± 0.06	**
LVEDP (%)	65.06 ± 6.89	65.49 ± 4.01	ns	82.65 ± 4.68	78.67 ± 9.69	ns
LVESP (%)	46.47 ± 5.75	46.76 ± 3.93	ns	55.40 ± 3.30	57.91 ± 8.55	ns

Table 3.20: Comparing the effect of trimetazidine (TMZ) treatment during the CAHF phase (lean males and lean luteal females) (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. Significance is expressed as *p<0.05; **p<0.01; ***p<0.001 versus lean males. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, Sig.: significance, ns: not significant.

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3.4.3.1.2 RAHF phase

Control lean males showed a respective 5.6-fold and 6.4-fold increase in RPP and $(dp/dt)_{max}$ from the previous CAHF phase. Lean control follicular females displayed increased (though not statistically significant) RPP and $(dp/dt)_{max}$ versus the lean males. There were no other differences between control lean males and control lean follicular females (Figure 3.13, Table3.21).

There was a substantial difference between males and females that were treated with TMZ during the previous CAHF phase. Here follicular TMZ-treated females exhibited substantially higher RPP ($p<0.001$), $(dp/dt)_{max}$ ($p<0.001$) and LVDevP ($p<0.001$) and this is due to the delayed treatment effect that was previously indicated. For the RAHF-treated animals, there were no significant differences (Figure 3.13, Table 3.21). Only males benefited from TMZ treatment during the RAHF phase, although this was not sufficient to provide superior performance versus the follicular females.

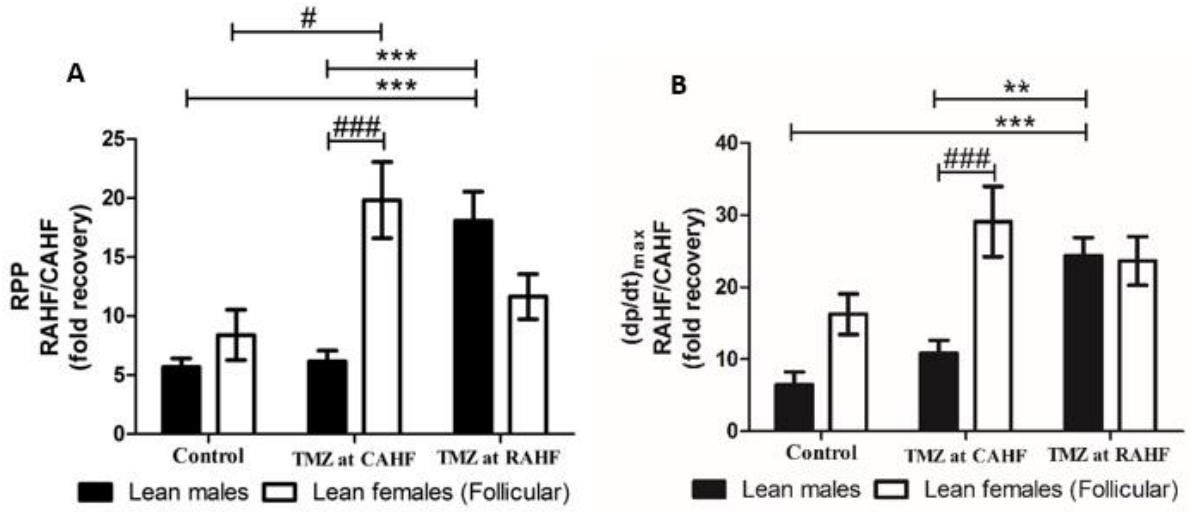

Figure 3.13: Effect of trimetazidine (TMZ) treatment during the RAHF phase on RPP and $(dp/dt)_{max}$ of lean males and lean follicular females. (A) RPP (B) $(dp/dt)_{max}$ (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a fold recovery from the previous CAHF phase. Significance is expressed as **p<0.01; ***p<0.001 versus TMZ at RAHF lean males and #p<0.05; ###p<0.001 versus TMZ at CAHF lean females. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure, RAHF: recovery acute heart failure.

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Comparing TMZ treatment for lean males vs. lean follicular females during RAHF						
	Control					
Parameters	**Males**			**Females**		**Sig.**
RPP (%)	568.43 ± 71.91			839.55 ± 213.56		ns
$(dp/dt)_{max}$ (%)	640.47 ± 184.14			1624.63 ± 283.02		ns
Heart rate (%)	210.13 ± 127.11			30.21 ± 4.14		ns
LVDevP (%)	1259.06 ± 485.90			2875.81 ± 725.74		ns
LVEDP (%)	201.85 ±36.57			140.87 ± 9.40		ns
LVESP (%)	211.06 ± 43.49			149.10 ± 10.88		ns
	TMZ at CAHF			**TMZ at RAHF**		
	Males	**Females**	**Sig.**	**Males**	**Females**	**Sig.**
RPP (%)	616.08 ± 91.27	1980.56 ± 321.98	***	1806.94 ± 245.55	1163.93 ± 191.79	ns
$(dp/dt)_{max}$ (%)	1077.15 ± 184.60	2911.24 ± 487.59	***	2435.16 ± 253.84	2364.91 ± 338.16	ns
Heart rate (%)	28.26 ± 1.48	27.94 ± 3.51	ns	29.74 ± 2.23	23.93 ± 2.00	ns
LVDevP (%)	2204.29 ± 355.33	8049.63 ± 1961.21	***	6025.70 ± 654.65	4918.75 ± 762.73	ns
LVEDP (%)	160.23 ± 9.38	139.35 ± 24.49	ns	158.26 ± 18.72	137.35 ± 3.71	ns
LVESP (%)	170.36 ± 11.07	152.26 ± 30.49	ns	171.80 ± 24.07	145.29 ± 4.73	ns

Table 3.21: Comparing the effect of trimetazidine (TMZ) treatment during the RAHF phase (lean males and lean follicular females) (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a percentage of CAHF. Significance is expressed as ***p<0.001 versus lean males. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, RAHF: recovery acute heart failure, Sig.: significance, ns: not significant.

Control lean males exhibited increased RPP and $(dp/dt)_{max}$ from the previous CAHF phase, while lean control luteal females showed a robust increase for both parameters (Figure 3.14). The latter values are higher (not significantly) than the recovery values for control follicular females (previously discussed) and contributes to the statistically significantly higher RPP ($p<0.05$) and $(dp/dt)_{max}$ ($p<0.05$) versus control lean males (Figure 3.14, Table 3.22).

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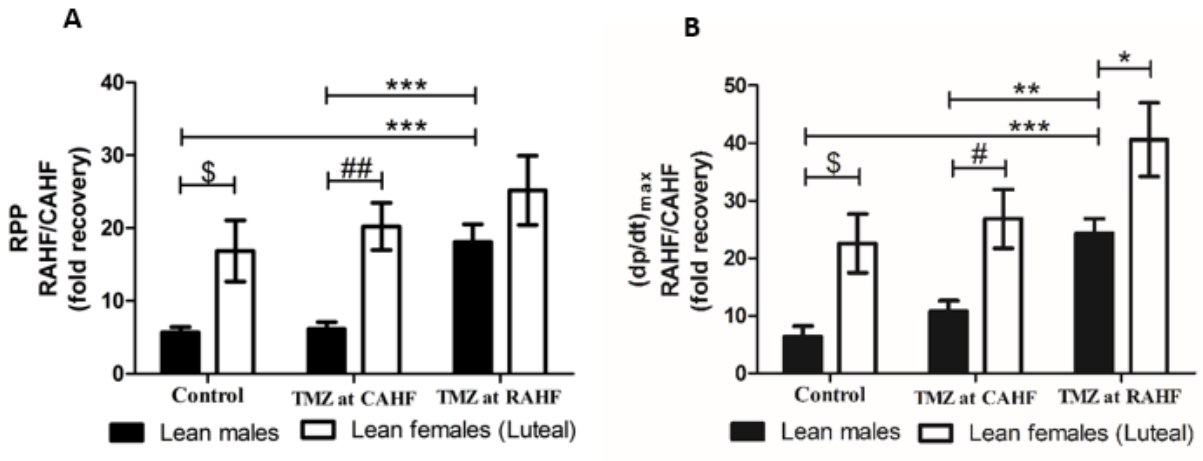

Figure 3.14: Effect of trimetazidine (TMZ) treatment during the RAHF phase on RPP and $(dp/dt)_{max}$ of lean males and lean luteal females. (A) RPP (B) $(dp/dt)_{max}$ (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a fold recovery from the previous CAHF phase. Significance is expressed as *p<0.05; **p<0.01; ***p<0.001 versus TMZ at RAHF lean males, #p<0.05; ##p<0.01 versus TMZ at CAHF lean males and $<0.05 versus control lean males. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure, RAHF: recovery acute heart failure.

As for follicular females, luteal females treated with TMZ during the CAHF phase displayed a higher RPP ($p<0.01$) and $(dp/dt)_{max}$ ($p<0.05$) compared to lean males. This picture emerges as a result of lean males only responding to TMZ treatment during the RAHF phase. However, this favorable response still did not provide lean males with superior heart functioning as RAHF-treated luteal females showed increased $(dp/dt)_{max}$ ($p<0.05$) versus the lean males (Figure 3.14, Table 3.22).

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Comparing TMZ treatment for lean males vs. lean luteal females during RAHF						
	Control					
Parameters	**Males**			**Females**		**Sig.**
RPP (%)	568.43 ± 71.91			1687.62 ± 421.02		*
$(dp/dt)_{max}$ (%)	640.47 ± 184.14			2255.71 ± 507.28		*
Heart rate (%)	210.13 ± 127.11			33.55 ± 4.86		ns
LVDevP (%)	1259.06 ± 485.90			6116.62 ± 1675.88		*
LVEDP (%)	201.85 ± 36.57			179.64 ± 10.82		ns
LVESP (%)	211.06 ± 43.49			199.59 ± 13.44		ns
	TMZ at CAHF			**TMZ at RAHF**		
	Males	**Females**	**Sig.**	**Males**	**Females**	**Sig.**
RPP (%)	616.08 ± 91.27	2021.86 ± 324.85	**	1806.94 ± 245.55	2519.13 ± 475.16	ns
$(dp/dt)_{max}$ (%)	1077.15 ± 184.60	2685.27 ± 513.67	*	2435.16 ± 253.84	4060.05 ± 639.68	*
Heart rate (%)	28.26 ± 1.48	34.18 ± 5.60	ns	29.74 ± 2.23	27.31 ± 1.41	ns
LVDevP (%)	2204.29 ± 355.33	6202.00 ± 1064.64	ns	6025.70 ± 654.65	9475.80 ± 1807.90	ns
LVEDP (%)	160.23 ± 9.38	176.24 ± 43.51	ns	158.26 ± 18.72	134.06 ± 4.27	ns
LVESP (%)	170.36 ± 11.07	194.08 ± 51.29	ns	171.80 ± 24.07	147.00 ± 6.88	ns

Table 3.22: Comparing the effect of trimetazidine (TMZ) treatment during the RAHF phase (lean males and lean luteal females) (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a percentage of CAHF. Significance is expressed as *p<0.05; **p<0.01; versus lean males. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, RAHF: recovery acute heart failure, Sig.: significance, ns: not significant.

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3.4.3.1.3 Summary

There were no significant differences between controls during the CAHF phase. With TMZ treatment during the CAHF phase, lean males and females displayed decreased heart function during the CAHF phase. However, during the RAHF phase control females exhibited increased recovery from the CAHF phase, with luteal control females showing the highest recovery. All females that were treated with TMZ during the CAHF phase showed greater functional recovery during RAHF compared to male counterparts. This is due to the delayed treatment effect observed for follicular females and also due to males only responding to TMZ treatment during the RAHF phase.

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3.4.3.2 Obese

3.4.3.2.1 CAHF phase

Control obese males showed RPP and $(dp/dt)_{max}$ values that were both 1.9% of the initial baseline whereas control obese females showed RPP and $(dp/dt)_{max}$ values that were respectively 3.6% and 2.5% of the initial baseline. The only significant difference between control obese males and females was that females displayed significantly reduced pressures. Females had moderately lower LVEDP ($p<0.01$) and LVESP ($p<0.01$). No differences were observed in functional parameters, but control obese females exhibited a tendency for higher RPP values ($p>0.05$) (Figure 3.15, Table 3.23).

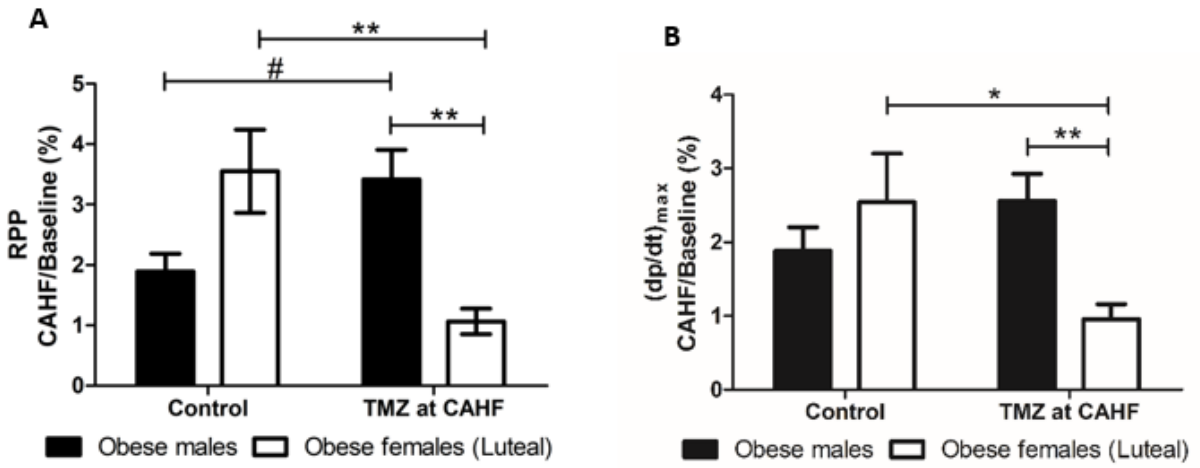

Figure 3.15: Effect of trimetazidine (TMZ) treatment during the CAHF phase on RPP and $(dp/dt)_{max}$ of obese males and females. (A) RPP (B) $(dp/dt)_{max}$ (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. Significance is expressed as *p<0.05; **p<0.01 versus TMZ at CAHF obese females and #p<0.05 versus control obese males. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure.

Pressure differences dissipated with TMZ treatment due to increasing pressures in the obese females (refer to previous data). TMZ treatment in the CAHF phase results in significant differences between obese males and females. Females had a significantly lower RPP ($p<0.01$) and $(dp/dt)_{max}$ ($p<0.01$) compared to obese males. This is due to the individual effects of TMZ treatment on the groups. Obese males positively benefit from TMZ treatment in the CAHF phase, whereas obese females respond negatively to the TMZ treatment (same as for lean females) (Figure 3.15, Table 3.23).

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Comparing TMZ treatment for obese males vs. females during CAHF						
	Control			TMZ at CAHF		
Parameters	**Males**	**Females**	**Sig.**	**Males**	**Females**	**Sig.**
RPP (%)	1.89 ± 0.29	3.55 ± 0.69	ns	3.42 ± 0.49	1.07 ± 0.21	**
$(dp/dt)_{max}$ (%)	1.89 ± 0.32	2.54 ± 0.66	ns	2.56 ± 0.37	0.95 ± 0.21	**
Heart rate (%)	382.43 ± 11.68	339.53 ± 48.09	ns	401.55 ± 22.64	335.46 ± 34.69	ns
LVDevP (%)	0.49 ± 0.07	1.40 ± 0.55	ns	0.86 ± 0.12	0.32 ± 0.05	**
LVEDP (%)	88.31 ± 3.07	47.29 ± 10.60	**	76.20 ± 5.19	87.69 ± 5.43	ns
LVESP (%)	62.29 ± 5.08	28.88 ± 6.73	**	56.83 ± 4.85	62.80 ± 4.86	ns

Table 3.23: Comparing the effect of trimetazidine (TMZ) treatment during the CAHF phase (obese males and females) (n=6). Data represents the last ten minutes of the CAHF phase and is expressed as a percentage of baseline. Significance is expressed as **p<0.01 versus obese males. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, Sig.: significance, ns: not significant.

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3.4.3.2.2 RAHF phase

Control obese males and females showed increased RPP and $(dp/dt)_{max}$ from the previous CAHF phase. There were no functional parameter differences between control obese males and females. Both obese males and females benefited from TMZ treatment during the RAHF phase and there were also no statistical differences between groups treated during the RAHF phase (Figure 3.16, Table 3.24).

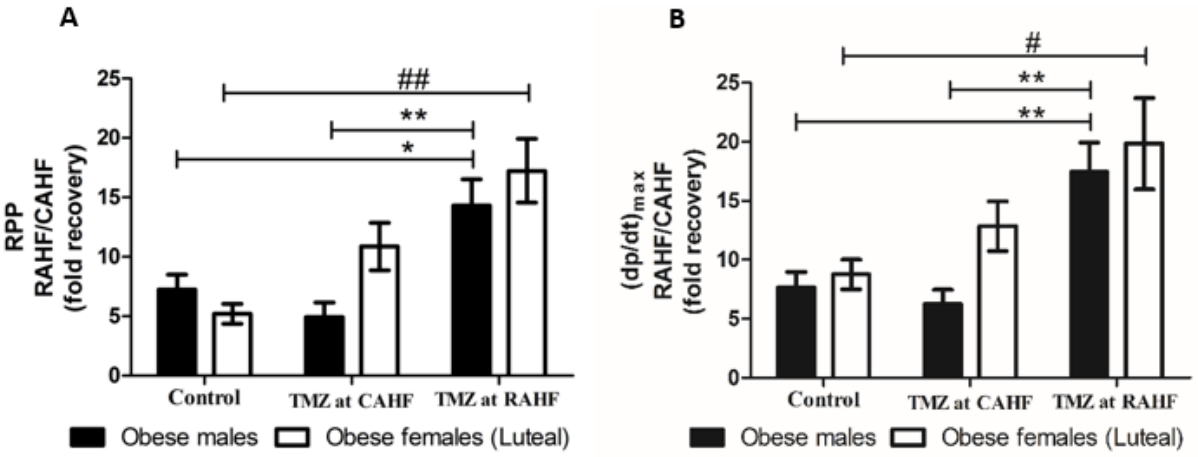

Figure 3.16: Effect of trimetazidine (TMZ) treatment during the RAHF phase on RPP and $(dp/dt)_{max}$ of obese males and females. (A) RPP (B) $(dp/dt)_{max}$ (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a fold recovery from the previous CAHF phase. Significance is expressed as *p<0.05; **p<0.01 versus TMZ at RAHF obese males and #p<0.05; ##p<0.01 versus TMZ at RAHF obese females. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure, RAHF: recovery acute heart failure.

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Comparing TMZ treatment for obese males vs. females during RAHF						
	Control					
Parameters	**Males**			**Females**		**Sig.**
RPP (%)	724.78 ± 124.26			519.68 ± 83.40		ns
$(dp/dt)_{max}$ (%)	763.84 ± 132.18			876.00 ± 125.34		ns
Heart rate (%)	31.75 ± 2.85			38.49 ± 10.56		ns
LVDevP (%)	2411.10 ± 458.16			1725.24 ± 397.04		ns
LVEDP (%)	142.48 ± 10.43			574.86 ± 308.07		ns
LVESP (%)	147.98 ± 11.77			521.49 ± 240.36		*
	TMZ at CAHF			**TMZ at RAHF**		
	Males	**Females**	**Sig.**	**Males**	**Females**	**Sig.**
RPP (%)	492.12 ± 122.78	1087.06 ± 199.76	ns	1430.89 ± 221.17	1722.53 ± 267.61	ns
$(dp/dt)_{max}$ (%)	624.06 ± 122.29	1284.62 ± 211.00	ns	1744.19 ± 249.20	1984.13 ± 387.63	ns
Heart rate (%)	30.17 ± 2.62	36.99 ± 3.84	ns	31.78 ± 2.38	32.55 ± 2.48	ns
LVDevP (%)	1678.49 ± 470.27	3199.25 ± 743.72	ns	4522.35 ± 742.07	5400.37 ± 887.21	ns
LVEDP (%)	158.98 ± 15.10	140.93 ± 11.62	ns	141.43 ± 9.98	126.83 ± 7.53	ns
LVESP (%)	164.74 ± 16.48	145.23 ± 12.91	ns	147.96 ± 12.18	132.12 ± 9.01	ns

Table 3.24: Comparing the effect of trimetazidine (TMZ) treatment during the RAHF phase (obese males and females (n=6). Data represents the last ten minutes of the RAHF phase and is expressed as a percentage of CAHF. Significance is expressed as *p<0.05 versus obese males. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, RAHF: recovery acute heart failure, Sig.: significance, ns: not significant.

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3.4.4 Summary of perfusion results

Although there were statistical differences found during the CAHF phase, these were of such small magnitude that it can be considered negligible. Control animals did not differ greatly in terms of the decline in functional parameters (Table 3.25), but some patterns emerged for the TMZ-treated groups. Here lean males showed no response to treatment while the obese counterparts benefited from TMZ treatment. However, TMZ treatment initiated during the CAHF phase was detrimental to lean and obese females early on at the end of the CAHF phase before RAHF.

For the RAHF phase, males displayed a maximum recovery of ~19% $(dp/dt)_{max}$ without any interventions. Lean females showed enhanced functional recovery without treatments, with follicular females recovering to ~31% (RPP) and luteal females to ~54% (RPP) of baseline. However, this inherent coping mechanism of the lean luteal females is lost with obesity as such animals reached less than half of this recovery (~19%, RPP)

Control animals						
	CAHF (% of Baseline)		RAHF (% of Baseline)		RAHF (Improvement from CAHF)	
	RPP	$(dp/dt)_{max}$	RPP	$(dp/dt)_{max}$	RPP	$(dp/dt)_{max}$
Lean males	3.1	3.0	17.36	19.2	5.6-fold	6.4-fold
Obese males	1.9	1.9	13.68	14.44	7.2-fold	7.6 fold
Lean follicular females	3.7	2.2	31.08	35.64	8.4-fold	16.2-fold
Lean luteal females	3.2	2.1	54.08	47.46	16.9-fold	22.6-fold
Obese females	3.6	2.5	18.72	22.00	5.2-fold	8.8-fold

Table 3.25: Comparing the effects of the *ex vivo* acute heart failure (AHF) model on control groups (n=6). The first RAHF column estimates the improvement as a percentage of the initial baseline (CAHF percentage x fold improvement in RAHF). RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure, RAHF: recovery acute heart failure.

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Lean and obese males responded favorably to TMZ treatment initiated during the RAHF phase compared to controls and animals treated during the CAHF phase. Obese females also benefited from TMZ treatment during the RAHF phase. Lean females exhibited a differential treatment response profile, i.e. lean follicular females showed a delayed TMZ treatment effect later-on, during the RAHF phase. However, lean luteal control females (displayed an inherent ability to) recovered remarkably from CAHF, while TMZ treatment had no significant effects during the RAHF phase.

These data show that there were no differences between lean and obese males, while lean females are at a clear functional recovery advantage over lean males. Here the luteal group displayed superior functional improvement and this is likely due to an innate coping mechanism. However, with the onset of obesity this advantage is lost.

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3.5 3-KAT and PDH expression

Treatment with TMZ during either of the phases showed no effect on 3-KAT nor PDH expression in any of the groups (Figure 3.17 and 3.18).

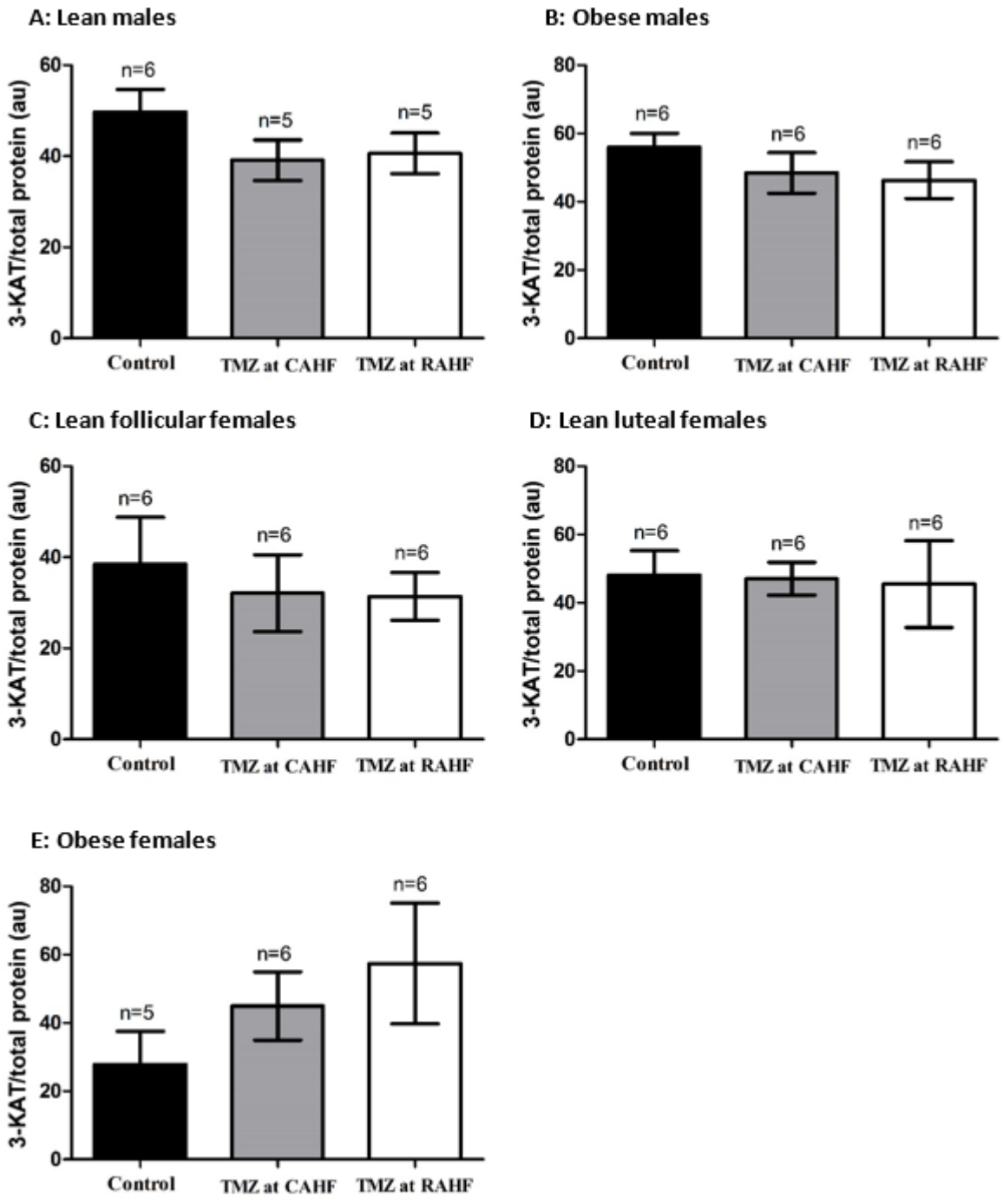

Figure 3.17: Effect of trimetazidine (TMZ) treatment on 3-KAT expression during the RAHF phase. (A) Lean males (B) Obese males (C) Lean follicular females (D) Lean luteal females (E) Obese females.

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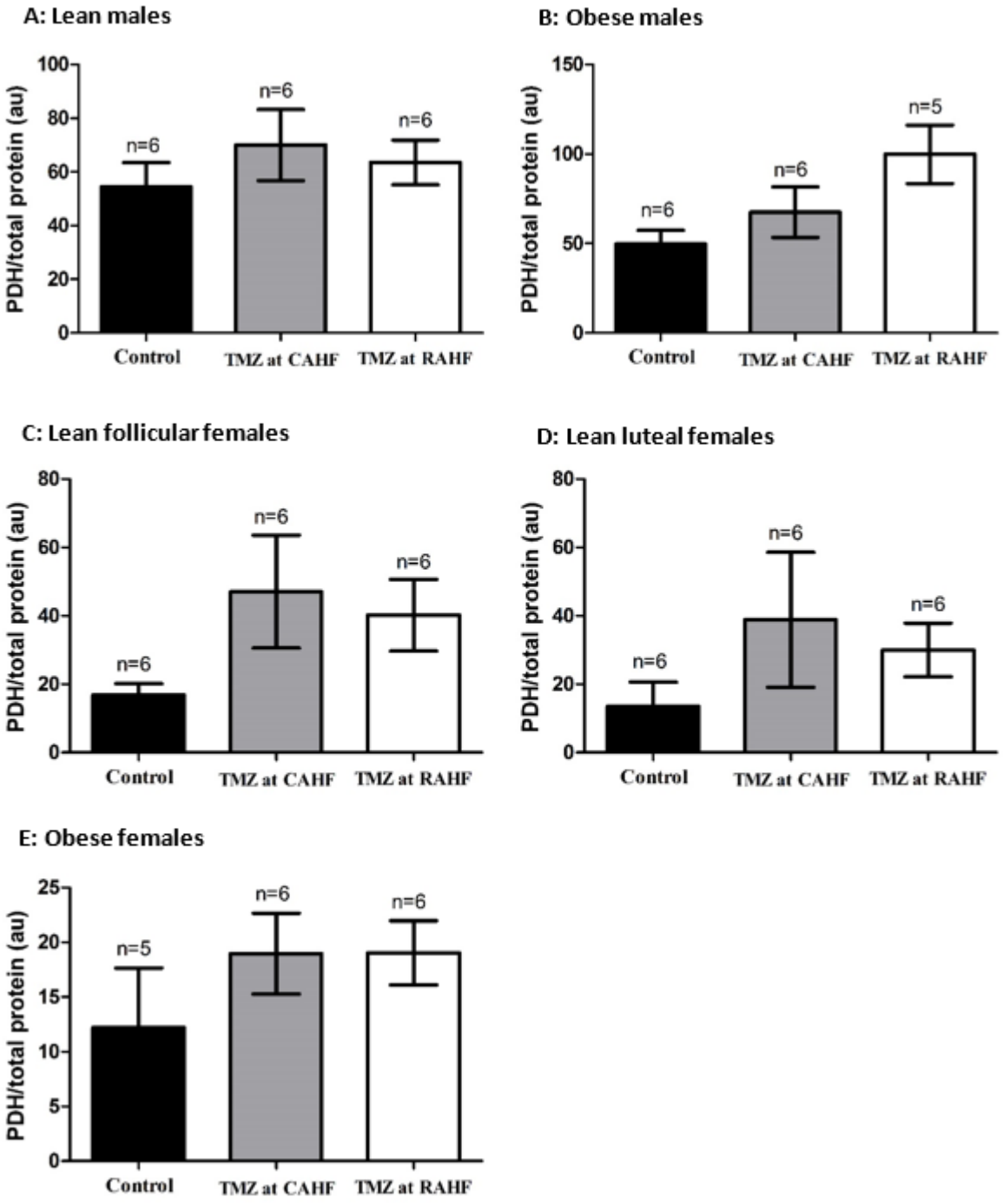

Figure 3.18: Effect of trimetazidine (TMZ) treatment on PDH expression during the RAHF phase. (A) Lean males (B) Obese males (C) Lean follicular females (D) Lean luteal females (E) Obese females.

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Chapter 4: Discussion

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4.1 Discussion

AHF – the lesser known "sister" of HF - is a complex clinical syndrome that varies extensively in terms of clinical presentations and pathophysiologic mechanisms underlying it (Metra *et al.*, 2010). ADHF is the most common primary diagnosis in heart disease patients admitted to African hospitals (Damasceno *et al*., 2007; Sliwa *et al.*, 2008). Moreover, African women suffering from AHFS are younger, with significant stress on the 20-29 year-old group, and are more prone to suffer from acute *de novo* HF (Ogah *et al.*, 2015; Sliwa & Mayosi, 2013). This type of AHF is also associated with increased risk of death compared to other forms (Follath *et al.*, 2011; Nieminen *et al.*, 2006; Tavazzi *et al.*, 2006), while co-morbidities such as diabetes and obesity further complicate the picture. In light of this bleak scenario, the optimization of cardiac metabolism emerges as a potentially novel therapeutic option for AHF treatment. For this study, we hypothesized that TMZ (partial FAO inhibitor) offers cardio protection during AHF under normal and obese-related diabetic conditions and that this may be influenced in a sex-dependent manner. The aims of this study were: (1) to establish a *de no* AHF mouse model; (2) to test whether simple FA-albumin filtering could replace the gold standard dialysis method; (3) to identify and establish the four phases of the estrous cycle in female mice; and (4) to evaluate the therapeutic value of TMZ under normal and obese-related diabetic conditions in both male and female mouse hearts subjected to a unique *ex vivo* model of AHF. We also tested the influence of the different phases of the follicular and luteal phases of the estrous cycle on TMZ efficacy and whether such treatment may be influenced in a sex-dependent fashion.

The main findings of this study are: (1) that our perfusion system resulted in significant decreases in both functional and pressure parameters during the CAHF phase, reflecting *de novo* AHF. Moreover, our model reflected partial recovery in the RAHF phase with modest increases in functional and pressure parameters although this was still significantly lower than baseline values; (2) the filtering FA-albumin method was useful and even marginally better than the gold standard dialysis method

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usually employed for Langendorff perfusion perfusates; (3) that lean (*db*/+) females showed normal estrous cycle phases namely proestrus, estrus (follicular phase), metestrus and diestrus (luteal phase). Obese females (*db*/*db*) did not, however, undergo the follicular phases (proestrus and estrus) and we did not find any differences in serum concentrations of estradiol and progesterone for any of the groups studied; and (4) TMZ treatment resulted in negligible changes to functional parameters during the CAHF phase, but significant results were obtained in terms of recovery function during the RAHF phase. Lean and obese males responded equally well to TMZ treatment. However, lean females (follicular and luteal phases) responded in distinct fashion, while obesity influenced females' response to TMZ administration. Due to the unique response pattern of the different estrous phases, sex differences were present only in lean groups.

4.1.1 Aim 1: Establish the *de novo* acute heart failure mouse model

In establishing our *ex vivo* mouse model of AHF, we based and modified the system on a previous acutely under perfused rat heart model (Deshpande *et al*., 2014; Deshpande, 2013; Deshpande *et al.,* 2010) where AHF was simulated by decreasing coronary perfusion pressure. This model is representative of hypotensive AHF patients who present with hypoperfusion, cardiogenic shock and low blood pressure (Filippatos & Zannad, 2007; Laribi *et al.*, 2012; Nieminen *et al.*, 2005). Lower pressure upon admission is inversely correlated with in-hospital mortality (Gheorghiade *et al.*, 2006). In agreement with the original Deshpande model, *de novo* AHF is induced in the isolated heart by decreasing the perfusion pressure from 100 cm to 20 cmH_2O and by altering metabolic substrates in the perfusate in order to 1) simulate circulating metabolite conditions of HF patients and 2) promote the upregulation of FA utilization during heart failure. We therefore introduced elevated FA (1.2 mM/L palmitic acid conjugated to 3% BSA) (Aasum & Larsen, 1997; Lopaschuk & Barr, 1997) and decreased the glucose concentration from 10 to 2.5 mM/L (Stanley & Chandler, 2002). Our perfusion system resulted in profound decreases in both functional and pressure parameters during the CAHF phase reflecting *de novo* AHF (Figure 3.4 and Table 3.2). The recovery phase is similarly manipulated by restoring the perfusion pressure back to 100 cmH_2O, restoring glucose to baseline levels (10

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mM/L) and by maintaining the high FA content. Our model reflected partial recovery during the RAHF phase (Figure 3.4 and Table 3.2) with modest increases in functional and pressure parameters that were still significantly lower than baseline values. A potential critique of this model may be the low recovery in functional parameters achieved. However, we are of the opinion that this model (with relatively weak recovery) is appropriate considering that hypoperfused patients with cardiogenic shock who have received hospital care display the highest cumulative mortality, reaching 53% and this is attributed to a high in-hospital mortality (Harjola *et al.*, 2010). Therefore a model that reaches a relatively high functional recovery would be irrelevant in studying a disease with such a deadly outcome.

4.1.2 Aim 2: Fatty acid-albumin filtering versus the gold standard dialysis method

When simulating the effects of high FA availability in a Langendorff isolated heart perfusion system, conjugated FAs are bound to BSA and here the dialysis method is routinely employed (Belke *et al*., 1999; Chatham *et al*., 1999; Gelinas *et al.*, 2008; Khairallah *et al.*, 2004; Lopaschuk & Barr, 1997; Ruiz *et al., 2015*) to minimize the contributions from impurities and contaminants in the conjugated perfusate. This method is, however, time-consuming and costly and we therefore decided to evaluate whether a simplified protocol, i.e. filtering (to remove the impurities and contaminants) would deliver comparable results. Our data revealed that the filtering method is equally useful, and even marginally better, than the gold standard dialysis method (Figure 3.3 and Table 3.1). This is supported by Gross (2009) who stated that dialysis is useful in a recirculating system where the heart contributes metabolic waste and if continued for a long period may result in metabolic "poisoning" of the system (Gross, 2009). It therefore stands to reason that in a non-circulating and relatively short protocol, the use of dialysis is not warranted. On the contrary, filtration of the perfusing buffer remains of the utmost importance as microaggregate impurities and/or salt precipitations can obstruct blood vessels (Gross, 2009; Ross-Ascuitto *et al.*, 1987; Ruiz *et al.*, 2015). Researchers may opt to utilize inline filters directly following the buffer reservoirs in the system or buffers can be filtered by vacuum filtering on the day of use. Of note, low protein binding filters

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must be utilized in order to prevent altering the composition of the perfusates. In summary, we found that filtering delivers more stable data over the duration of the experiment, with no changes in functional parameters. Moreover, it was a faster and more cost-effective method to apply in our laboratory. The filtering method was therefore employed for all perfusion experiments completed in this study.

4.1.3 Aim 3: Identify and establish the four phases of the estrous cycle in female mice

In order to evaluate whether the estrous cycle may impact on TMZ efficacy during an AHF event, we initially identified the different phases of the estrous cycle by vaginal cytology (Caligioni, 2009; Marcondes *et al.,* 2002). The short estrous cycle (lasting 4-5 days) makes the mouse an ideal model to investigate influences of the reproductive cycle. After exposing the females to male pheromones to ensure regular cycling (MacDonald *et al.,* 2014; Whitten, 1958), the lean (*db*/+) females showed normal estrous cycle phases (Figure 3.1) namely proestrus, estrus (follicular phase), metestrus and diestrus (luteal phase). However, obese females (*db*/*db*) did not undergo the follicular phases (proestrus and estrus), but rather displayed metestrus and diestrus acyclicity, a phenomenon common in this transgenic mouse strain (Bates *et al.*, 2003; Johnson & Sidman, 1979; Robertson *et al.*, 2010). Leptin is an adipokine that is secreted by adipose tissue and its function is critical to body weight and composition, insulin sensitivity and reproduction (Leibel *et al*., 1997). It also functions as a feedback signal or "fat reporter" (Wurtman, 1996) to the nervous system to regulate energy homeostasis and serves as an index of stored energy in the body (Maffei *et al.*, 1995). When leptin feedback is interrupted (Montague *et al.*, 1997), a state of negative energy balance is sensed despite actual energy stores available, causing hyperphagia (primarily by increased meal sizes) (de Jong-Nagelsmit *et al.,* 1986), attenuated energy expenditure (Yen & Acton, 1972) and a decreased ability to utilize stored energy (Gao & Horvath, 2008). Leptin receptor deficiencies result in obesity and insulin resistant diabetes (Coleman, 1978), both states of energy turmoil. There is a strong hypothalamic link between energy balance and reproduction (Hill *et al*., 2008) and parallel cross talk with estrogen (Gao & Horvath, 2008) that leads to reproduction abnormalities.

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In addition, we also evaluated whether such phases corresponded with the expected hormonal fluctuations of estradiol and progesterone in serum. Here we found no differences in estradiol and progesterone levels between the four phases of the estrous cycle in lean females (Figure 3.2), nor between animal groups including obese females and lean/obese males (data not shown). Past studies reported a very specific pattern in that the follicular phase is dominated by estradiol as it increases throughout proestrus and reaches a maximum at the onset of ovulation in estrus (Fata *et al.*, 2001; Walmer *et al*., 1992; Wood *et al*., 2007). Moreover, the luteal phase is dominated by progesterone. Our non-significant results could be explained by a lack of sensitivity of commercial ELISA kits as these have been shown to be lacking in accuracy, sensitivity and reliability (Haisenleder *et al.,* 2011; Handelsman *et al.,* 2011). In addition, analytical assessments of sex steroids are generally difficult to accurately and consistently measure (Bielohuby *et al.,* 2012; McNamara *et al.*, 2010; Parks & Howlett, 2013). Both liquid chromatography–tandem mass spectrometry (LC–MS/MS) (McNamara *et al*. 2010) and gas chromatography–tandem mass spectrometry (GC–MS/MS) (Haisenleder *et al.,* 2011) assays are ideal as these provide exceptional accuracy, sensitivity, specificity and capability. Of note, although we were unable to accurately detect such differences in animals here studied, it should be borne in mind that such physiological differences do indeed manifest.

4.1.4 Aim 4: Evaluate the therapeutic value of TMZ under various conditions

In order to pursue our last aim, we decided to not only evaluate the recovery (RAHF phase) as is typically done, but to also assess heart function during the ‘’assault’’ or CAHF phase. Here mice did not differ significantly in terms of the decline in functional parameters (Table 3.25) during the CAHF phase. However, TMZ treated hearts showed small, although statistically significant differences in functional parameters during the CAHF phase (Table 3.25). We speculate that such alterations are due to the length of the phase and that if it was continued any longer then such hearts would not have recovered at all. A shorter phase could possibly induce less damage and could deliver statistical

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results of a larger magnitude. The functional assessment during the RAHF phase produced more compelling results.

Our results showed that lean and obese males recovered in a similar manner (Figure 3.6) at both treatment time points, i.e. TMZ treatment during the CAHF and RAHF phases, respectively. Groups receiving TMZ during the CAHF phase showed no statistical differences in heart function during their recovery (Table 3.6 & 3.7) and was expected (Figure 4.1) as TMZ treatment during the CAHF phase would partially inhibit 3-KAT and consequently increase glucose utilization (Banach *et al*., 2008; Dezsi, 2014; Huqi *et al.,* 2015; Kantor *et al.*, 2000; Lopaschuk *et al.,* 2003; Marzilli & Huqi, 2011). However, during the CAHF phase glucose available for utilization is exceptionally low and TMZ treatment shifts metabolism away from the only plentiful metabolite that is available to the heart at this point. Treatment during the RAHF phase, however, resulted in significant functional recovery (Table 3.6 and 3.7). Metabolic therapies (e.g. TMZ) are extensive areas of research and been successful for CVD treatment with the latter attributed to the promotion of glucose utilization restoring homeostasis and alleviating the detrimental effect of increased FAO (Banach *et al.*, 2008; Fang *et al.*, 2012; Fragasso *et al.*, 2008; Lopaschuk, 2001; Lopaschuk *et al.*, 2003; Sabbah & Stanley, 2005). For example, TMZ increased mechanical heart function in *db/db* mice by attenuating FAO-mediated lipotoxicity and oxidative stress, and also suppressing and preventing the development of DCM (L *et al.,* 2010; Ussher *et al.*, 2014). This was confirmed in obese humans, where TMZ improved myocardial efficiency by decreasing FAO (Bucci *et al.*, 2012). Our results also demonstrate that obesity does not influence TMZ efficacy for AHF treatment in male mice (Figure 3.6 and Table 3.8).

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Baseline (Stabilization)	Critical Acute Heart Failure (CAHF)	Recovery Acute Heart Failure (RAHF)
Normal glucose No FAs **(Normal Fuel)**	Low glucose FAs **(Push towards FAO)**	Normal glucose FAs **(Choice in fuel)**
Normal pressure Normal perfusion Paced	Low pressure Low perfusion Not paced	Normal pressure Normal perfusion Paced
Expected function: Normal	Expected function: Critically low	Expected function: Increased function
	TMZ treatment: Restore equilibrium back to glucose utilization	**TMZ treatment:** Favours the equilibrium towards glucose utilization
		

Figure 4.1: Acute heart failure (AHF) *ex vivo* model – the principle. FAs: fatty acids, FAO: Fatty acid β-oxidation.

Lean females in the two different phases of the estrous cycle responded in unique ways (Figure 3.8). Here lean follicular females recovered optimally when treated with TMZ during the CAHF phase, unlike when there was no significant recovery when the hearts were treated during the RAHF phase (Table 3.12). The lean luteal females were the only group to exhibit inherent superior functional recovery and was also the only group that was not significantly affected by TMZ treatment for any of the treatment time points (Table 3.13). This led us to conclude that this treatment difference may be due to the effect of sex hormones, i.e. the follicular phase (dominated by estrogen) and the luteal phase (characterized by progesterone) (MacDonald *et al.*, 2014; Walmer *et al.*, 1992; Wood *et al.*, 2007). However, our ELISA results did not reflect this. The lean follicular females was the only group to show significant recovery when TMZ was administered during the CAHF phase. As previously discussed, our model shifts metabolism towards FAO during CAHF, but TMZ treatment would

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encourage less desirable utilization of glucose (Figure 4.1) that is severely decreased. An explanation of the beneficial effects of TMZ could be that estrogen increases FAO and decreases glucose utilization (Campbell & Febbraio, 2001), thus allowing the myocardium to utilize the high FAs during this phase. In support, females seem to be better adapted to utilize FA and may also be protected against increased FA levels compared to males (Konhilas, 2010).

Our results revealed that the lean luteal females did not respond to TMZ treatment. Research in sex differences in pharmacokinetics and pharmacodynamics (Soldin & Mattison, 2009; Soldin *et al*., 2011) demonstrated that increased sex hormones in women can alter hepatic enzyme activities leading to fluctuations in drug accumulation and/or elimination. Drug metabolism can be altered during the latter stages of the luteal phase as estrogen is a substrate for CYP3A (Bigos *et al.,* 2009; Soldin *et al.*, 2011). This may explain the lack of response to TMZ in our model as the biotransformation of TMZ is speculated to involve isoenzymes of CYP3A (Jackson *et al.*, 1996; Simon *et al.*, 1997). The findings generated in this study also demonstrate that lean luteal females have an inherent "coping mechanism" to better handle the AHF event, despite the lack of response to TMZ treatment. This may be attributed to the protective effects of progesterone on the cardiovascular system (Prior, 2014), e.g. it can assist in cardiac repair by enhancing fibroblast regeneration and differentiation (Dubey *et al.,* 1998). Moreover, progesterone displays greater counter-regulatory responses in response to adrenergic stimulae (Moldovanova *et al.*, 2008), it can antagonize vasoconstriction (Barbagallo *et al.*, 2001), is associated with increased blood flow (Mather *et al*., 2000) and can decrease arrhythmias and the risk of sudden cardiac death (Odening *et al.*, 2012). We propose that any of these factors could influence the apparent innate protection observed in the lean luteal females. Intriguingly, this inherent cardio protection is lost with obesity in luteal females across all the treatment groups (Figure 3.10 and Table 3.18). Obese females exhibited the same treatment pattern as their lean counterparts, but at lower values (Figure 3.10 and Table 3.17). Furthermore, obese luteal females responded to TMZ treatment the same as the males and benefitted only when it was added during the RAHF phase. We speculate that the treatment effect

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in females may be affected by the degree of obesity as obese female mice displayed a 20% increase in body weight compared to 10% for males (Bates *et al.*, 2003). In addition, specific sex differences in body fat content and protein binding have been shown to affect distribution of drugs (Ochs *et al.*, 1981; Soldin & Mattison, 2009).

Our results revealed that sex differences in TMZ efficacy as a therapy for AHF is only present in lean groups (Figure 3.13 & 3.14 and Table 3.21 & 3.22). There were no differences in functional recovery between obese males and females (Figure 3.16 and Table 3.24), showing that obesity is detrimental to both sexes. Sex differences are present between the lean males, and both follicular and luteal lean females. Sex differences in pharmacokinetics and pharmacodynamics is an exceptionally common and prevailing phenomenon and has resulted in a vast research field (Bigos *et al.*, 2009; Soldin & Mattison, 2009; Soldin *et al.*, 2011). In summary, differences arise due to sex differences in drug absorption (gastric and hepatic enzyme activities, transporter proteins, and handling of drugs or metabolites), drug distribution (body fat composition and cardiac output), drug metabolism (cytochrome P450 group, hepatic and extra-hepatic metabolism) and drug elimination. We propose that the distinct sex differences found in the two estrus phases may be due to varying treatment patterns as a result of sex hormone differences.

To our knowledge we are the first group to demonstrate a sex difference in the therapeutic value of TMZ (Vitale *et al.*, 2013) and to specifically show an involvement of the estrous cycle in this process. Moreover, as far as we are aware this is the first study to investigate this within the context of AHF (Belardinelli & Purcaro, 2001; Di Napoli *et al.,* 2005).

Western Blot analysis of protein expression of 3-KAT and PDH did not reveal any alterations with TMZ treatment (Figure 3.17 & 3.18). Previous perfusion studies evaluated enzyme activity (Kantor *et al.*, 2000; Lopaschuk *et al.*, 2003) of these enzymes and found positive results. However, the Lopaschuk *et al.* (2003) study was conducted on normal male Sprague-Dawley rats and the concentration of TMZ used was 100 µM (we used 5 µM). In addition, Kantor *et al.* (2000) also

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utilized normal male Sprague-Dawley rats and perfused with relatively low palmitic acid levels (0.4 mM, compared to 1.2 mM employed by this study) bound to BSA. Others reported changes in expression levels, although this was for a chronic model of TMZ treatment (8 weeks) for streptozotocin-diabetic rats (Onay-Besikci *et al.*, 2007). We believe that the discrepancies in drug and FA concentration and the choice of animal model (rats versus mice; type 1 versus type 2 diabetes) may account for such differences. Additionally, the short drug exposure (25 minute perfusion phase) may also explain why it is too early to detect changes in myocardial protein expression.

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4.2 Conclusion

AHFS has received limited attention to date and limitations of previous study designs make it difficult to derive meaningful sex-based comparisons. The generic nature of interventions show that current therapies are not tailored/designed specifically for AHF. The optimization of cardiac metabolism (especially TMZ) emerges as a novel and worthy therapeutic option to investigate for the treatment of AHF. We have successfully established a novel *ex vivo* mouse AHF model and also shown that the less costly and more time-effective filtering method can replace dialyses in Langendorff perfusions. We confirmed that obese females (*db*/*db*) display metestrus acyclicity and diestrus acyclicity. We showed that TMZ elicits negligible effects on heart function during the CAHF phase, but that it results in significant recovery for both males and obese females when administered during the RAHF phase. Sex differences are also present in with such therapy as the estrous cycle influences the therapeutic utility of TMZ.

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4.3 Limitations and future research

Although we demonstrated that females respond to an AHF insult in a unique way in this pilot study, our model only mimics the subset of AHF that males are more prone to suffer (by hypoperfusion) (Table 1.3). Future work should focus on setting up two models of AHF, one for each classification of AHF that each gender is more prone to experience. Additionally, this study could not investigate how obese females in the follicular phase would respond. Future studies could also include investigation into the two remaining sex hormones (follicle stimulating hormone and luteinizing hormone) and females could be divided into all four phases of the estrous cycle.

Our current perfusion model only utilized two exogenous substrates (palmitate and glucose) to mimic the metabolic alteration during a cardiac insult and thus neglecting the effects of other energy sources namely lactate, pyruvate and ketone bodies. Furthermore, the relative substrate contributions (and how altered) should be ascertained. Future studies should not only investigate fuel substrate oxidation, but also uptake and esterification in addition to enzyme activity and expression.

Future work could progress towards an *in vivo* model of AHF where cardiac failure is rapidly induced in a known and characterized manner. Live 3D imaging could also be performed on animals to evaluate cardiac function during the assault and during recovery phases. Ideally, future work should utilize a diet-induced obesity/diabetic model as this is more plausible and clinically relevant. Our model also represented an assault in extreme obese and diabetic states that are untreated for diabetes. Thus future studies should include groups to represent treated diabetic individuals as this will further strengthen efforts to translate basic biomedical research findings into the clinical context.

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Addendum A

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Approved with Stipulations

Date: 02-Apr-2014

PI Name: Breedt, Emilene ES

Protocol #: SU-ACUM14-00010

Title: Perturbed metabolic and signalling pathways in the female diabetic heart during the different stages of the estrous cycle

Dear Emilene Breedt, the Initial Application submission was reviewed on 02-Apr-2014 by Research Ethics Committee: Animal Care and Use via committee review procedures and was approved on condition that the following stipulations are adhered to:

1. The researcher is requested to motivate and explain why 25 animals are needed for calibration?
2. Is it possible to have less than 8 animals per group? For statistical significance 5-6 could be sufficient. Please clarify.
3. How often will mice have vaginal swabs
4. How often and when will the animals be che[illegible]
5. Animal transport: how will the animals be tr[illegible]ported? Details have to be given on cages, method and what precautions will be used to minimize stress to the animals.

APPROVED

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

Please remember to use your protocol number, SU-ACUM14-00010 on any documents or correspondence with the REC: ACU concerning your research protocol.

If you have any questions or need further help, please contact the REC: ACU secretariat at WABEUKES@SUN.AC.ZA or 0218089003.

Sincerely,

Winston Beukes

REC: ACU Secretariat

Research Ethics Committee: Animal Care and Use

DIE UNIVERSITEIT VAN STELLENBOSCH
08 MAY 2014
THE UNIVERSITY OF STELLENBOSCH

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Addendum B

Krebs-Henseleit buffer for mouse heart - stabilization phase

Stock solutions: Concentrated (500x or 1000x) solutions are made up and stored in the fridge.

Working solutions and final concentrations: On the day of use, alliquates from the stock solutions are diluted to the final working buffer (final concentrations).

Stock solutions for mouse Krebs:

Reagents can be purchased from Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States.

Potassium chloride (KCl) (#P9333) (2.01 M): stock = 150 g in 1 L, add 10 ml to the 5 L

Monosodium phosphate (NaH_2PO_4)(#S8282) (1.16 M): stock = 140 g in 1 L, add 5 ml to the 5 L

Disodium ethylenediaminetetraacetic acid (EDTA.$2H_2O$) (#D2900000) (0.5 M): stock = 190 g in 1 L, add 5 ml to the 5 L

Magnesium chloride ($MgCl_2$).$6H_2O$ (#M2670) (2.5 M): stock = 240 g in 1 L, add 5 ml to the 5 L

Calcium chloride ($CaCl_2$).$2H_2O$ (#C5080) (3.3 M): stock = 370 g in 1 L, add 5 ml to the 5 L

* Sodium chloride (NaCl); Sodium bicarbonate ($NaHCO_3$)

	5 Liter	4 Liter	2 Liter
Final concentrations:	Measure ½-¾ of final volume distilled H_2O accurately into flask then weigh out and add:		
NaCl (#S7653) 118 mM	34.48 g	27.58 g	13.79 g
$NaHCO_3$ (#S6297) 24 mM	10.1 g	8.08 g	4.04 g
Add alliquates of stock solutions to make up final concentrations:	Stir until all is dissolved then add:		
KCl 4.02 mM	10 ml	8 ml	4 ml
NaH_2PO_4 1.16 mM	5 ml	4 ml	2 ml
Disodium EDTA.$2H_20$ 0.5 mM	5 ml	4 ml	2 ml
$MgCl_2$.6H20 2.5 mM	5 ml	4 ml	2 ml
$CaCl_2$ 3.3 mM	5 ml	4 ml	2 ml
Final glucose concentration:	Stir until all is mixed and check that no $CaCl_2$ precipitates out. Then add the glucose and stir.		
Glucose (#G7528) 10 mM	9.0 g	7.2 g	3.6 g
	Mix well and top up to final volume		
	pH (Basic 20, Crison, Hach Lange Spain, SLU, Barcelona, Spain) to7.4		

Table A1: Krebs-Henseleit buffer for mouse heart - stabilization phase.

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Palmitic acid stock solution (120 mM) for the CAHF and RAHF phase

Add 2.99 g sodium carbonate (Na_2CO_3) and 6.15 g palmitic acid, make up to 200 ml final volume with 38% ethanol. Stir at approximately 60 ˚C. Cover the flask to prevent evaporation. On the day of use, heat the stock solution as it solidifies at low temperatures.

Critical -and recovery AHF phase buffer for mouse heart

Make up buffer standard Krebs-Henseleit buffer as stipulated above except for the glucose and addition of the BSA (3%)/1.2 mM palmitate conjugate as follows:

	2 Liter	1 Liter	0.5 Liter
CAHF phase: (low glucose + BSA/palmitate conjugation)			
Glucose 2.5 mM	Add 0.9 g	Add 0.45 g	Add 0.225 g
RAHF phase: (normal glucose + BSA/palmitate conjugation)			
Glucose 10 mM	Add 3.6 g	Add 1.8 g	Add 0.9 g
Both Phases: BSA (3%)/1.2 mM palmitate conjugation according to Lopaschuk and Barr 1997			
BSA	60 g	30 g	15 g
Palmitic acid (from stock solution)	20 ml	10 ml	5 ml

Table A2: Krebs-Henseleit buffer for mouse heart – CAHF and RAHF phase.

BSA (Fraction V, Roche Ltd., Basel, Switzerland)

Palmitic acid (#P0500, Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States)

Sodium carbonate (#S7795, Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States)

Ethanol (#E7023, Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States)

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Addendum C

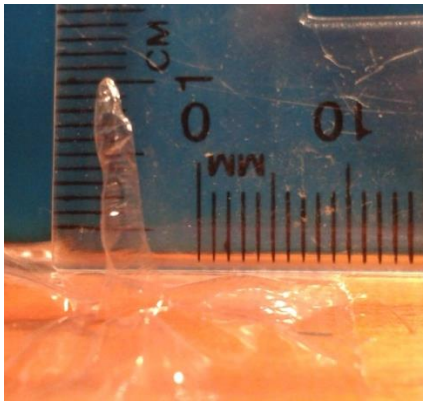

Figure A1: Cling wrap cone for the construction of mouse ventricular balloons. Slowly pull the cling wrap over the point of the needle, about 1-2 cm down. Hold the cling wrap in close proximity to the needle shaft as this helps to not only stretch the cling wrap to its maximum but also to "shape" the cling wrap better.

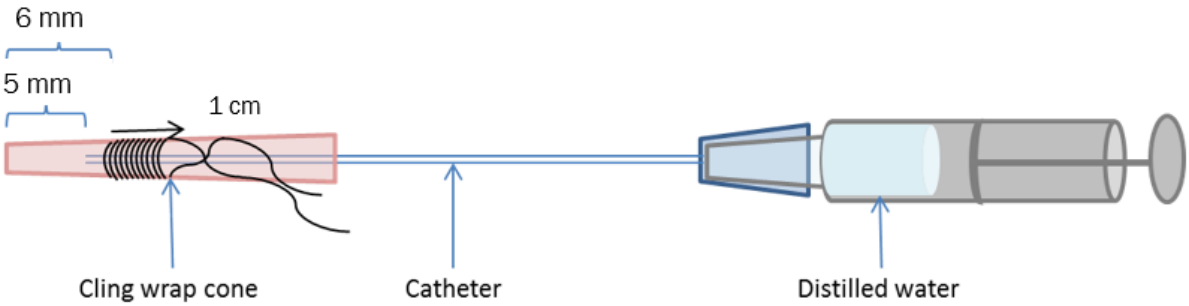

Figure A2: Constructing mouse ventricular balloons. With the catheter attached to the syringe, pull degassed distilled water up into the syringe, making sure there is no air bubbles in the catheter. Put the tip of the water-filled catheter into the cone shape. Leave at least a 5 mm space between the tips of the cling wrap cone and the catheter. Wind the invisible thread neatly around the catheter: leave a tail of thread at the back (syringe side) and start winding from the tip of the catheter upward for about 1 cm. The starting point of the "coil" should be close to the tip of the catheter – max 1 mm from the tip. Knot off sufficiently to prevent leaks.

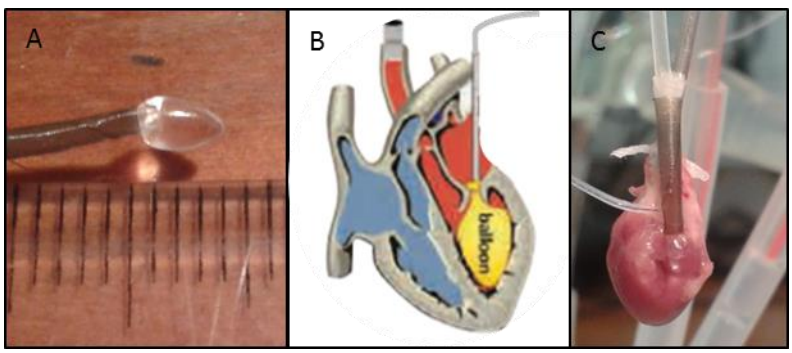

Figure A3: Mouse ventricular balloon. This balloon shape assures that the whole ventricle is filled. Sharp corners will cause damage to the ventricles, causing the heart to fail on the rig.

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Addendum D

A.D.1 Preparation of RIPA buffer (modified Radio Immuno PrecipitAtion buffer) and protein extraction

Prepare 100 ml modified RIPA base buffer as follows:

1. Add 790 mg Tris base (65.21 mM) (#T6791, Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States) to 75 ml dH_2O. Add 900 mg NaCl (154.00 mM) and stir the solution until all solids are dissolved. Adjust the pH to 7.4.

2. Add 10 ml of 10% Tergitol-type NP-40 (1%) (#74385, Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States) to the solution.

3. Add 2.5 ml of 10% Na-deoxycholate (0.25%) (#D6750, Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States) and stir until solution is clear.

4. Add 1 ml of 100 mM EDTA (1mM) (#D2900000, Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States) to the solution. Adjust the volume of the solution to 100 ml using a graduated cylinder.

5. Ideally, the remaining protease and phosphatase inhibitors should be added to the solution on the same day you are running the assay. Store at 2-8° C.

Modified RIPA with inhibitors: Add the appropriate amount of each protease inhibitor to the RIPA base so that the final concentrations are:

Reagents can be purchased from Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States:

- Phenylmethylsulfonyl fluoride (PMSF) (#7626): 1 mM
- Leupeptin (#L2884) and benzamidine (#B6505): 1 µg/ml
- Aprotonin (#A1153): 1 µg/ml
- Pepstatin (#P5318): 10 µg/ml
- Activated Sodium orthovanadate (Na_3VO_4) (#S6508): 1 mM

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- Sodium fluoride (NaF) (#S7920): 1 mM
- Note: PMSF is extremely unstable in aqueous solutions with a half-life of approximately 30 minutes and it should be added immediately before use.

Protein extraction

Wrap each piece of tissue sample in little squares of aluminium foil (foil folded over 4 x) and place in liquid nitrogen. Pour a small amount of liquid nitrogen into mortar and pulverize the frozen sample. Carefully unwrap the foil and tip the powdered tissue (keeping it cool) into tubes containing RIPA buffer (with inhibitors) – aim to make homogenates that are 1:2 w/v tissue:lysis buffer). Transfer the homogenates to fresh pre-chilled microfuge tubes and keep on ice. Once all samples have been homogenized, sonicate (Sp-4000 ultrasonic liquid processor, Misonix, New York, United States) for 15 seconds each and then centrifuge at 12000 rpm (3,312 x g) for 15 minutes at 4°C. Transfer the supernatants (protein lysates) to fresh pre-chilled tubes and thereafter perform protein determination. Freeze samples at -20°C if not running gels on same day.

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A.D.2 Bradford protein determination

Stock solution 5x concentration:

Dilute 500 mg Coomassie Brilliant blue G250 (#27815, Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States) in 250 ml 95% ethanol. Add 500 ml phosphoric acid (W290017, Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States) and mix thoroughly. Adjust the volume to 1 L with dH_2O. Filter (filter paper, 240 mm, Munktell, Bärenstein, Germany) solution and store at 4°C.

Working solution:

Dilute the stock solution in a 1:5 ratio (100 ml:500 ml) with dH_2O. Filter with 2 filter papers. The filtered working solution should be a light brown color and is light sensitive.

Method:

Thaw 2 ml of 200 µg/ml BSA stock solution and the protein samples. Work on ice at all times. Prepare and mark 2 ml Eppendorf tubes (eppis) for the standard curve. Mark duplicates.

Prepare standard curve as follows:

BSA	dH_2O	Concentration
0	100 µl	Blank
10 µl	90 µl	2 µg
20 µl	80 µl	4 µg
40 µl	60 µl	8 µg
60 µl	40 µl	12 µg
80 µl	20 µl	16 µg
100 µl	0 µl	20 µg

Table A3: Standard curve for Bradford protein determination.

- For the samples, place 95 µl dH_2O into each sample tube and add 5 µl of protein sample to appropriate duplicate tubes and vortex (Vortex mixer vm-300, Gemmy Industrial Corp., Taipei, Taiwan) briefly.
- Add 900 µl Bradford working solution to each tube and vortex again.

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- Incubate tubes at room temp for at least 5 minutes until they equilibrate to room temperature. The reaction is stable for up to 60 minutes.
- Let the spectrophotometer (Cecil CE 2021, Cecil instruments, Cambridge, England) warm up to calibrate, then set wavelength to 595 nm.
- Read absorbances of standards and samples
- If absorbance of samples falls outside the range of the highest standard, dilute with RIPA buffer and read again – remember to multiply result by dilution.
- Draw up the standard curve (Microsoft Office 2010, Excel, Redmond, Washington, United States) and thereafter determine the unknown protein values (in µg).

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A.D.3 Sample preparation

Laemmli's loading buffer

dH_20	3.8 ml
0.5 M Tris- Hydrogen chloride (HCl), pH 6.8 (#T6666 Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States)	1.0 ml
Glycerol (#818709, Merck Millipore Co., Darmstadt, Germany)	0.8 ml
10 % (w/v) sodium dodecyl sulfate (SDS) (#L3771, Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States)	1.6 ml
0.05 % (w/v) Bromophenol blue (#108122, Merck Millipore Co., Darmstadt, Germany)	0.4 ml

Table A4: Laemmli's loading buffer.

Protocol:

1. Make a working solution of Laemmli's sample buffer by adding 850 µl Laemmli's stock buffer solution to 150 µl β-mercaptoethanol (#1610710, Bio-Rad Laboratories Inc., Hercules, California, Unites States) (work in fume hood) and mixing thoroughly on the vortex.
2. Calculate the appropriate volume of each sample to give equal loading amounts (previously calculated on the excel sheet) and calculate the number of sample sets needed.
3. Add the appropriate volumes of working solution of Laemmli's, RIPA buffer (as calculated) and protein sample to each appropriate tube (work in fume hood).
4. Close tubes and punch small hole in lid and heat samples on heating block (Incu Block, Labnet International, Inc., Edison, New Jersey, United States), at 95˚C for 5 minutes.
5. Vortex and spin down contents briefly (± 10 sec) and place on ice immediately.
6. Samples can now either be stored at -20 ^{o}C for future use, or be loaded onto the gels.

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A.D.4 Casting and running SDS-PAGE gels

Preparation:

Collect and clean the clean spacer and short plates (#1653311, Bio-Rad Laboratories Inc., Hercules, California, United States) and assemble them in the casting stand with clamps kit (#1658052, Bio-Rad Laboratories Inc., Hercules, California, Unites States). Check that the setup is not leaking by pouring ethanol between the plates. Remove the ethanol hereafter.

Make up working solutions of running buffer and TBS-T:

Reagents can be purchased from Sigma-Aldrich Co. LLC., St. Louis, Missouri, United States:

10x running buffer (2 liter)	**10x TBS (2 liter)**
60.6 g Tris	48.4 g Tris
288 g Glycine (#50046)	160 g NaCl
20 g SDS	Dissolve in 1200 ml, adjust the pH to 7.6 and make up to final volume.
In 1.5 liter d.H_2O, first dissolve the Tris and glycine and then add the SDS. pH to 8.6. Make up to final volume.	
1x working solution (1 liter)	1x working solution TBS-T (1 liter)
Mix 900 ml d.H_2O and 100 ml 10x stock solution.	Mix 900 ml d.H_2O and 100 ml 10x stock solution, 1 ml Tween-20 (P1379) and mix well.

Table A5: Working solutions of running buffer and TBS-T.

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Preparing and casting the stain-free gels:

Make up the desired amount of resolving gel end pour this between the plates up to the top green bar on the casting stand, making sure to remove all the bubbles. Leave this to set for 40-60 minutes.

Gel constituent	**6%**	**8%**	**10%**	**12%**	**15%**
dH_20	8.625 ml	7.875 ml	7.125 ml	5.7 ml	5.245 ml
40% Acrylamide (#A718) solution	2.25 ml	3.0 ml	3.75 ml	4.5 ml	5.625 ml
1.5 M Tris-HCl, pH 8.8	3.75 ml	3.75 ml	3.75 ml	3.75 ml	3.75 ml
10% w/v SDS	150 µl	150 µl	150 µl	150 µl	150 µl
10% w/v APS (#09913)	150 µl	150 µl	150 µl	150 µl	150 µl
TCE (#T54801)	75 µl	75 µl	75 µl	75 µl	75 µl
* TEMED (#T702) (add last!)	12 µl	9 µl	6 µl	6 µl	6 µl

Table A6: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) resolving gel.

* Tetramethylethylenediamine (TEMED)

When the resolving gel is set, prepare the 4% stacking gel as follows with the same reagents.

Gel constituent	**4%**
dH_20	4.84 ml
40% Acrylamide solution	1.0 ml
0.5 M Tris-HCl, pH 6.8	2.0 ml
20% w/v SDS	40 µl
10% w/v APS	40 µl
TEMED (add last)	8 µl

Table A7: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stacking gel

Pour between the plates, on top of the set resolving gel and insert the desired comb (#1653359, Bio-Rad Laboratories Inc., Hercules, California, United States), making sure that it corresponds to the thickness of the plates that are used. Let it set for approximately 30 minutes.

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Running the gels:

Fetch and clean the electrophoresis system (#1658004, Mini-PROTEAN Tetra Vertical Electrophoresis Cell, 4-gel, Bio-Rad Laboratories Inc., Hercules, California, United States)

- Carefully remove the comb then remove the plates from the casting stands and frames and place in the U-shaped adaptor with the short plate facing inwards. Insert this assembly into the loading system and close the latches to form a buffer dam in the center.
- Place the assembly in the gel tank and fill with cold 1x running buffer and wash the wells with running buffer.
- Add 7.5 µl of the stained and 5 µl unstained protein marker to each gel.
- Add the appropriate amount (calculated from Bradford protein quantification) of sample slowly into the appropriate lane.
- Place the green lid with appropriate leads (black to black: red to red) on top of the tank and connect to the power pac (#4006038, Power Pac 1000, Bio-Rad Laboratories Inc., Hercules, California, Unites States).
- Run at 110 V (constant), 400 mA for about 2 hours to allow samples to migrate through gel - until the sample dye front and smallest standard of the protein marker reaches the bottom of the gel. Switch off power and disconnect electrodes. Remove gel plates from the tank.

Activating the stain free whole protein:

- Gently separate the plates, remove the gel and place it on the tray of the ChemiDoc (ChemiDoc MP System, Bio-Rad Laboratories Inc., Hercules, California, United States) and close the drawer.
- Open the accompanying Image Lab program (Image Lab Software, version 5.0, Bio-Rad Laboratories Inc., Hercules, California, Unites States), select “new protocol”, under “Application” choose “Protein gels” and “Stain free”. Select the 1 minute activation protocol, position your gel and run the protocol. Save the image.

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- Immediately proceed to electrotransfer step.

Protein transfer, whole protein imaging and primary anti-body:

- Open the "transfer pack" (#1704157, Trans-Blot Turbo Midi PVDF Transfer Packs, Bio-Rad Laboratories Inc., Hercules, California, Unites States)
- Following the enclosed instructions, assemble the transfer "sandwich" on the tray of the transfer system (# 1704155, Trans-Blot Turbo Transfer Starter System, Bio-Rad Laboratories Inc., Hercules, California, Unites States) and roll out the bubbles. Lock the lid in place.
- Set the system to transfer on the standard "SD 25V 1A" program for 30 minutes.
- Place the membrane in methanol (#822283, Merck Millipore Co., Darmstadt, Germany) for 30 seconds and let it air dry completely.
- Wash the membrane 3 x 5 minutes in TBS-T and place on the tray of the ChemiDoc once more. In Image Lab select "new protocol", under "Application" choose "Blots" and "Stain free". Select the preferred optimization option for your membrane, position your membrane and run the protocol. Save the image. This imaging step is to check that the transferring was successful but will also be the image that your protein of interest will be normalized to.
    - Block for 2 hours on a shaker (GyroTwister, LabNet, New Jersey, United states) in 5% milk or 5% BSA – made up in TBS-T. Wash 3 x 5 minutes in TBS-T.
    - Place membrane in 50 ml falcon tube (50050, Conical tube, SPL life sciences, Gyeonggi-do, Korea) containing 5 ml of primary antibody diluted in TBS-T (dilution determined by product data sheet and optimization) Place on rotator (H5600 Revolver, LabNet, New Jersey, United states) in 4°C walk-in fridge overnight.

Secondary anti-body and protein of interest imaging:

- Remove the membrane from primary antibody (save antibody – freeze at -20°C) and wash 3 x 5 minutes in TBS-T.

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- Place membrane in 50 ml falcon tube containing 5 ml of secondary antibody diluted in TBS-T (dilution determined by product data sheet and optimization). Place on rotator (SRT9 roller mixer, Stuart Bibby scientific Ltd., Staffordshire, United Kingdom) for 1 hour at room temperature.
- Remove the membrane from secondary antibody (save antibody – freeze at -20°C) and wash the membrane 3 x 5 minutes in TBS-T.
- Prepare ECL (Bio-Rad Clarity Western ECL substrate, Bio-Rad Laboratories Inc., Hercules, California, Unites States) substrate in a 1:1 ratio of solutions A:B in a foil covered tube. Pour off the TBS-T from membrane and add ECL, spreading evenly over surface of membrane.
- Place the membrane on the tray of the Chemidoc, taking care to remove bubbles and close the drawer.
- In Image Lab select “New protocol”, under “Application” choose “Blot” and “Chemi”. Set the imaging area to 12 x 9. Now either choose one of the automatic exposure options (intense or faint bands) or manually set the exposure time (optimization) until one of your bands (protein of interest) become overexposed (red). Position your membrane and run the protocol. Save the image.

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A.D.5 Densitometry and normalization to whole protein:

In the Image Lab program, open both the whole protein stain free blot image (taken right after transferring) and your chemiluminescent protein of interest image.

- Select “File” and “Create multichannel image”. Drag the stain free blot image to channel 1 and the chemiluminescent image to channel 2. Click “OK” and the “RGB” button.
- In the “Analysis tool box”, select “Lane and bands”. Click on the stain free blot to make it the active image. In the “Lanes” tab, you can now either automatically detect the lanes or draw them manually. Either way you can use the lane adjustment tools to position the lanes. Set the “Disc size” to 70 mm. Copy (Ctrl+C) these lanes and paste (Ctrl+V) these lanes onto the chemiluminescent image. Select the “Bands” tab and then “Detect bands”, select the appropriate sensitivity and click “Detect”.
- Go back to the “Analysis tool box” and select “Normalization”. Under “Normalization channel” select “Stain free blot”. Select the radio button for “Total lane protein”.
- Go back to the “Analysis tool box” and select “MW analysis tools”. On the stain free image, check the boxes under the lanes where you have loaded your protein standards. Under “Standards” select the protein ladders that you have used.
- On the main toolbar, select “Analysis table” and click the “export analysis table to Excel” button. All calculations have been performed by the software.

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A.D.6 Western blot images

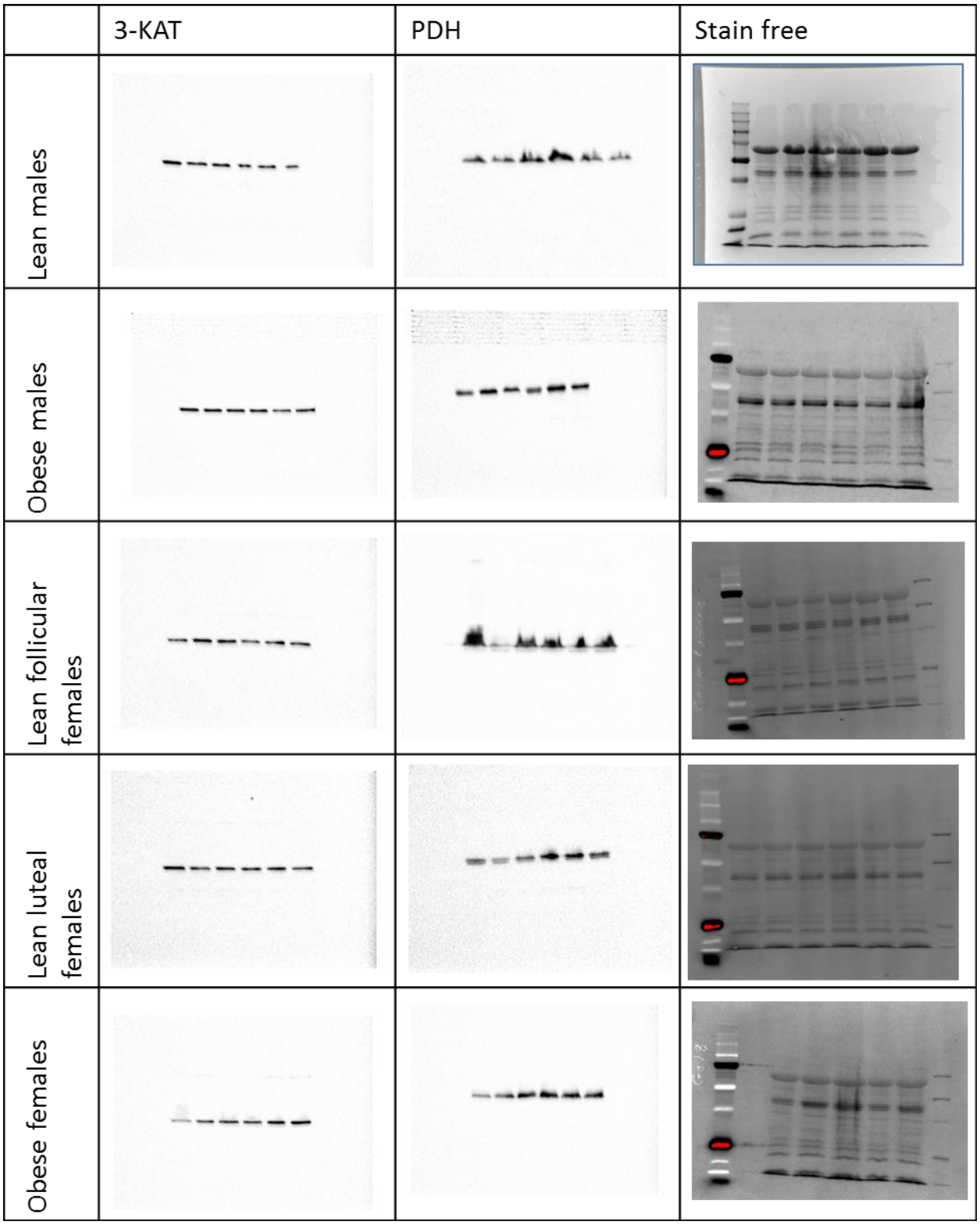

Table A8: Representative Western blot images. 3-KAT: 3-keotacyl-CoA thiolase, PDH: Pyruvate dehydrogenase

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Addendum E

Filter			
	Baseline	10-15 min	25-30 min
RPP	5955.00 ± 291.00	6679.00 ± 321.30	8012.00 ± 477.00
$(dp/dt)_{max}$	570.90 ± 38.52	632.10 ± 38.82	710.30 ± 46.73
Heart rate	394.30 ± 1.62	395.40 ± 2.03	411.30 ± 10.90
LVDevP	15.10 ± 0.72	16.88 ± 0.76	19.55 ± 1.32
LVEDP	150.90 ± 27.78	149.20 ± 27.49	144.70 ± 27.24
LVESP	166.00 ± 28.13	166.10 ± 27.81	164.30 ± 27.12
Dialysis			
	Baseline	10-15 min	25-30 min
RPP	4384.00 ± 1198.00	5194.00 ± 1448.00	6110.00 ± 1389.00
$(dp/dt)_{max}$	382.60 ±82.55	499.30 ± 123.7	602.60 ± 113.40
Heart rate	396.40 ± 0.94	369.30 ± 15.07	338.70 ± 25.85
LVDevP	11.04 ± 3.00	14.56 ± 4.23	17.90 ± 3.59
LVEDP	175.20 ± 26.60	173.00 ± 26.00	170.50 ± 25.81
LVESP	186.20 ± 24.71	187.60 ± 25.03	188.40 ± 24.82

Table A9: Raw perfusion results for filter and dialysis groups. Units of raw values are as follows: LVESP, LVEDP and LVDevP in mmHg, $(dp/dt)_{max}$ in mmHg/s, RPP in mmHg/min and heart rate in beats per minute HR: heart rate, RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, TMZ: trimetazidine, SEM: standard error of the mean.

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	Baseline	CAHF Phase	SAHF Phase
RPP	16590.00 ± 2213.00	484.60 ± 95.76	2514.00 ± 336.30
$(dp/dt)_{max}$	1105.00 ± 133.00	46.16 ± 13.47	193.00 ± 28.03
Heart rate	395.60 ± 1.11	1088.00 ± 322.20	471.00 ± 51.88
LVDevP	41.93 ± 5.59	1.68 ± 0.86	5.60 ± 0.91
LVEDP	92.78 ± 9.45	63.08 ± 10.44	110.30 ± 11.40
LVESP	134.70 ± 12.10	64.75 ± 10.50	115.90 ± 10.62

Table A10: Raw perfusion results for establishing the *ex vivo* mouse model of acute heart failure. Units of raw values are as follows: LVESP, LVEDP and LVDevP in mmHg, $(dp/dt)_{max}$ in mmHg/s, RPP in mmHg/min and heart rate in beats per minute HR: heart rate, RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, TMZ: trimetazidine, SEM: standard error of the mean.

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Control			
	Baseline	CAHF phase	RAHF phase
RPP	16590.44 ± 2212.90	484.56 ± 95.76	2514.09 ± 336.30
$(dp/dt)_{max}$	1105.22 ± 133.05	46.16 ± 13.47	192.98 ± 28.03
Heart rate	395.58 ± 1.11	1088.27 ± 322.23	470.99 ± 51.88
LVDevP	41.93 ± 5.59	1.67 ± 0.86	5.60 ± 0.91
LVEDP	92.78 ± 9.45	63.08 ± 10.44	110.32 ± 11.40
LVESP	134.71 ± 12.10	64.75 ± 10.50	115.92 ± 10.62
TMZ at CAHF			
	Baseline	CAHF phase	RAHF phase
RPP	18622.26 ± 923.04	656.12 ± 77.05	3723.18 ± 307.75
$(dp/dt)_{max}$	1301.16 ± 159.63	28.92 ± 2.84	290.97 ± 30.18
Heart rate	394.70 ± 2.62	1609.02 ± 64.39	451.87 ± 19.67
LVDevP	47.20 ± 2.39	0.41 ± 0.06	8.26 ± 0.70
LVEDP	98.25 ± 8.99	80.85 ± 8.15	125.85 ± 5.83
LVESP	145.45 ± 7.98	81.26 ± 8.15	134.11 ± 5.55
TMZ at RAHF			
	Baseline	CAHF phase	RAHF phase
RPP	20129.06 ± 766.12	255.50 ± 31.34	4269.98 ± 350.86
$(dp/dt)_{max}$	1360.37 ± 62.13	13.80 ± 1.49	322.22 ± 18.38
Heart rate	388.82 ± 4.58	1426.27 ± 70.26	420.95 ± 30.39
LVDevP	51.83 ± 2.20	0.18 ± 0.01	10.23 ± 0.75
LVEDP	137.43 ± 27.59	117.71 ± 27.42	164.83 ± 28.77
LVESP	189.26 ± 27.72	117.88 ± 27.42	175.07 ± 28.40

Table A11: Raw perfusion results lean males (n=6). Units of raw values are as follows: LVESP, LVEDP and LVDevP in mmHg, $(dp/dt)_{max}$ in mmHg/s, RPP in mmHg/min and heart rate in beats per minute HR: heart rate, RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, TMZ: trimetazidine, SEM: standard error of the mean.

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Control			
	Baseline	CAHF phase	RAHF phase
RPP	18548.97 ± 1839.97	344.72 ± 61.40	2390.40 ± 429.70
$(dp/dt)_{max}$	1202.68 ± 92.22	22.02 ± 3.41	163.84 ± 31.96
Heart rate	392.13 ± 0.29	1499.71 ± 46.49	477.71 ± 51.20
LVDevP	47.30 ± 4.68	0.22 ± 0.03	5.20 ± 1.07
LVEDP	125.95 ± 20.76	111.81 ± 19.02	149.81 ± 16.70
LVESP	173.25 ± 17.36	112.04 ± 19.01	155.02 ± 16.30
TMZ at CAHF			
	Baseline	CAHF phase	RAHF phase
RPP	16057.13 ± 1850.13	558.33 ± 119.89	2290.67 ± 295.98
$(dp/dt)_{max}$	1091.55 ± 135.09	26.52 ± 3.48	154.70 ± 20.30
Heart rate	394.81 ± 1.62	1585.91 ± 91.25	469.16 ± 26.66
LVDevP	40.62 ± 4.58	0.35 ± 0.07	4.99 ± 0.70
LVEDP	118.90 ± 13.61	93.72 ± 15.01	138.07 ± 14.08
LVESP	159.53 ± 15.40	94.07 ± 15.05	143.06 ± 14.19
TMZ at RAHF			
	Baseline	CAHF phase	RAHF phase
RPP	19034.68 ± 1325.03	221.62 ± 28.10	2977.63 ± 411.68
$(dp/dt)_{max}$	1312.90 ± 49.54	11.44 ± 0.51	194.79 ± 23.32
Heart rate	396.27 ± 3.91	1487.03 ± 126.66	462.03 ± 32.82
LVDevP	48.00 ± 3.25	0.15 ± 0.01	6.56 ± 0.90
LVEDP	147.43 ± 21.66	128.37 ± 20.53	171.55 ± 20.72
LVESP	195.44 ± 20.24	128.52 ± 20.54	178.11 ± 20.67

Table A12: Raw perfusion results obese males (n=6). Units of raw values are as follows: LVESP, LVEDP and LVDevP in mmHg, $(dp/dt)_{max}$ in mmHg/s, RPP in mmHg/min and heart rate in beats per minute HR: heart rate, RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, TMZ: trimetazidine, SEM: standard error of the mean.

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Control			
	Baseline	CAHF phase	RAHF phase
RPP	16289.71 ± 1894.48	873.11 ± 336.58	4269.78 ± 535.97
$(dp/dt)_{max}$	1116.99 ± 139.07	25.42 ± 6.51	344.07 ± 43.13
Heart rate	402.02 ± 5.73	1494.89 ± 183.64	416.93 ± 14.47
LVDevP	40.37 ± 4.47	0.62 ± 0.28	10.27 ± 1.31
LVEDP	160.59 ± 22.71	131.62 ± 23.69	175.16 ± 23.37
LVESP	200.96 ± 24.35	132.24 ± 23.83	185.44 ± 24.01
TMZ at CAHF			
	Baseline	CAHF phase	RAHF phase
RPP	17079.71 ± 2149.35	250.92 ± 20.01	4862.54 ± 697.89
$(dp/dt)_{max}$	1237.81 ± 153.30	14.08 ± 1.05	403.26 ± 66.48
Heart rate	390.32 ± 2.98	1522.87 ± 132.96	403.41 ± 20.07
LVDevP	43.84 ± 5.63	0.16 ± 0.01	12.27 ± 2.04
LVEDP	163.06 ± 23.69	150.97 ± 25.29	180.47 ± 22.85
LVESP	206.91 ± 23.11	151.13 ± 25.28	192.75 ± 22.93
TMZ at RAHF			
	Baseline	CAHF phase	RAHF phase
RPP	14958.69 ± 1039.71	376.23 ± 54.90	4006.36 ± 439.18
$(dp/dt)_{max}$	1140.17 ± 91.60	15.50 ± 2.11	340.82 ± 32.23
Heart rate	389.18 ± 3.20	1595.62 ± 72.03	377.88 ± 25.19
LVDevP	38.45 ± 2.74	0.24 ± 0.04	10.52 ± 0.91
LVEDP	170.02 ± 18.01	137.85 ± 15.55	186.88 ± 16.67
LVESP	208.48 ± 16.91	138.09 ± 15.57	197.41 ± 16.62

Table A13: Raw perfusion results lean follicular females (n=6). Units of raw values are as follows: LVESP, LVEDP and LVDevP in mmHg, $(dp/dt)_{max}$ in mmHg/s, RPP in mmHg/min and heart rate in beats per minute HR: heart rate, RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, TMZ: trimetazidine, SEM: standard error of the mean.

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Control			
	Baseline	CAHF phase	RAHF phase
RPP	14579.51 ± 1651.19	464.67 ± 150.92	4846.39 ± 538.99
$(dp/dt)_{max}$	1187.81 ± 140.37	24.74 ± 6.58	399.25 ± 53.39
Heart rate	396.82 ± 5.16	1293.74 ± 136.56	401.48 ± 11.61
LVDevP	36.94 ± 4.33	0.46 ± 0.22	12.23 ± 1.58
LVEDP	93.53 ± 13.35	63.19 ± 13.12	106.70 ± 13.30
LVESP	130.47 ± 14.09	63.66 ± 13.06	118.93 ± 14.00
TMZ at CAHF			
	Baseline	CAHF phase	RAHF phase
RPP	16921.82 ± 1817.65	267.17 ± 42.46	4847.59 ± 510.36
$(dp/dt)_{max}$	1203.42 ± 144.97	15.48 ± 2.36	381.00 ± 49.60
Heart rate	399.93 ± 2.45	1390.17 ± 123.26	442.67 ± 23.43
LVDevP	42.20 ± 4.30	0.18 ± 0.01	11.10 ± 1.21
LVEDP	132.32 ± 25.15	109.52 ± 24.56	149.73 ± 23.76
LVESP	174.53 ± 23.56	109.71 ± 24.55	160.83 ± 23.35
TMZ at RAHF			
	Baseline	CAHF phase	RAHF phase
RPP	20754.22 ± 1354.62	323.84 ± 65.10	6695.62 ± 817.95
$(dp/dt)_{max}$	1483.47 ± 115.19	14.17 ± 1.50	540.25 ± 70.12
Heart rate	394.65 ± 2.84	1529.01 ± 56.64	414.26 ± 12.71
LVDevP	52.61 ± 3.48	0.21 ± 0.04	16.41 ± 2.20
LVEDP	166.19 ± 22.14	144.39 ± 22.56	188.83 ± 23.36
LVESP	218.81 ± 23.66	144.60 ± 22.54	205.24 ± 22.82

Table A14: Raw perfusion results lean luteal females (n=6). Units of raw values are as follows: LVESP, LVEDP and LVDevP in mmHg, $(dp/dt)_{max}$ in mmHg/s, RPP in mmHg/min and heart rate in beats per minute HR: heart rate, RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, TMZ: trimetazidine, SEM: standard error of the mean.

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Control			
	Baseline	CAHF phase	RAHF phase
RPP	13983.18 ± 2410.90	444.18 ± 70.35	2112.03 ± 326.94
$(dp/dt)_{max}$	965.00 ± 167.91	20.68 ± 3.96	163.30 ± 23.56
Heart rate	384.16 ± 11.08	1307.46 ± 195.11	407.14 ± 16.44
LVDevP	36.34 ± 5.87	0.39 ± 0.09	5.13 ± 0.68
LVEDP	59.83 ± 6.86	29.40 ± 7.94	77.71 ± 6.30
LVESP	96.18 ± 6.52	29.79 ± 7.87	82.84 ± 6.71
TMZ at CAHF			
	Baseline	CAHF phase	RAHF phase
RPP	19291.76 ± 1953.15	190.55 ± 27.56	1937.27 ± 274.60
$(dp/dt)_{max}$	1375.89 ± 122.23	11.96 ± 1.76	140.43 ± 14.91
Heart rate	396.26 ± 2.20	1327.96 ± 136.30	466.16 ± 24.94
LVDevP	48.64 ± 4.86	0.14 ± 0.01	4.09 ± 0.48
LVEDP	127.47 ± 16.21	113.69 ± 17.15	152.03 ± 17.19
LVESP	176.12 ± 16.83	113.84 ± 17.16	156.12 ± 17.01
TMZ at RAHF			
	Baseline	CAHF phase	RAHF phase
RPP	17091.87 ± 1871.77	189.09 ± 24.45	3341.99 ± 684.70
$(dp/dt)_{max}$	1171.45 ± 125.58	10.93 ± 0.66	228.29 ± 54.29
Heart rate	391.76 ± 3.73	1569.94 ± 85.98	503.02 ± 26.42
LVDevP	43.49 ± 4.56	0.12 ± 0.02	6.95 ± 1.62
LVEDP	167.01 ± 24.39	151.84 ± 27.39	185.39 ± 25.35
LVESP	210.51 ± 24.74	151.96 ± 27.39	192.35 ± 25.30

Table A15: Raw perfusion results obese females (n=6). Units of raw values are as follows: LVESP, LVEDP and LVDevP in mmHg, $(dp/dt)_{max}$ in mmHg/s, RPP in mmHg/min and heart rate in beats per minute HR: heart rate, RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, TMZ: trimetazidine, SEM: standard error of the mean.

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Baseline raw values								
	N1	N2	N3	N4	N5	N6	Average	SEM
RPP	14893.15	15381.79	7705.60	23327.67	20770.48	17463.97	16590.44	2212.90
$(dp/dt)_{max}$	1229.52	1105.66	561.73	1490.52	1310.62	933.25	1105.217	133.05
HR	393.20	395.25	394.84	395.69128	393.71	400.80	395.58	1.11
LVDevP	37.88	38.92	19.52	58.95	52.75	43.57	41.93	5.59
LVEDP	107.96	49.63	92.55	112.99	88.26	105.30	92.78	9.45
LVESP	145.84	88.55	112.07	171.94	141.02	148.87	134.71	12.10
LVEDP	107.96	49.63	92.55	112.99	88.26	105.30	92.78	9.45
CAHF phase raw values								
RPP	428.10	876.43	257.07	443.21	274.27	628.28	484.56	87.41
$(dp/dt)_{max}$	67.96	51.20	98.58	27.45	18.34	13.42	46.16	12.30
HR	117.95	1760.14	51.25	1690.17	1542.58	1367.54	1088.27	294.16
LVDevP	3.63	0.50	5.02	0.26	0.18	0.46	1.67	0.78
LVEDP	69.53	17.39	62.44	96.55	66.06	66.49	63.08	9.53
LVESP	73.16	17.89	67.45	96.81	66.24	66.95	64.75	9.59
RAHF phase raw values								
RPP	2299.72	3830.25	1981.00	1625.15	2179.72	3168.71	2514.09	307.00
$(dp/dt)_{max}$	195.82	322.35	160.09	119.48	178.02	182.11	192.98	25.59
HR	394.71	398.83	407.72	553.60	376.55	694.53	470.99	47.36
LVDevP	5.83	9.60	4.86	2.94	5.79	4.56	5.60	0.83
LVEDP	114.09	66.63	94.50	150.08	121.35	115.31	110.32	10.41
LVESP	119.92	76.23	99.36	153.01	127.14	119.87	115.92	9.69
Raw RAHF/Raw CAHF (as a percentage)								
RPP	537.20 %	437.03 %	770.60 %	366.67 %	794.75 %	504.34 %	568.43 %	0.66
$(dp/dt)_{max}$	288.13 %	629.61 %	162.40 %	435.27 %	970.46 %	1356.92 %	640.46 %	1.68
HR	334.66 %	22.66 %	795.53 %	32.75 %	24.41 %	50.79 %	210.13 %	1.16
LVDevP	160.52 %	1928.69 %	96.87 %	1119.47 %	3255.74 %	993.06 %	1259.06 %	4.44
LVEDP	164.10 %	383.10 %	151.35 %	155.45 %	183.69 %	173.42 %	201.85 %	0.33
LVESP	163.92 %	426.12 %	147.30 %	158.06 %	191.93 %	179.04 %	211.06 %	0.40

Table A16: Calculating raw perfusion results into a percentage ratio. Data represents the last ten minutes of each phase. Units of raw values are as follows: LVESP, LVEDP and LVDevP in mmHg, $(dp/dt)_{max}$ in mmHg/s, RPP in mmHg/min and heart rate in beats per minute. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, LVDevP: left ventricular developed pressure, LVEDP: left ventricular end diastolic pressure, LVESP: left ventricular end systolic pressure, CAHF: critical acute heart failure, RAHF: recovery acute heart failure, SEM: standard error of the mean.

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	Control animals						CAHF treated animals						RAHF treated animals			
	CAHF (% of Baseline)		RAHF (% of Baseline)		RAHF (Fold Improvement from CAHF)		CAHF (% of Baseline)		RAHF (% of Baseline)		RAHF (Fold improvement from CAHF)		RAHF (% of Baseline)		RAHF (Fold improvement from CAHF)	
	RPP	$(dp/dt)_{max}$	RPP	$(dp/dt)_{max}$	RPP	$(dp/dt)_{max}$	RPP	$(dp/dt)_{max}$	RPP	$(dp/dt)_{max}$	RPP	$(dp/dt)_{max}$	RPP	$(dp/dt)_{max}$	RPP	$(dp/dt)_{max}$
Lean males	3.1	3	17.4	19.2	5.6	6.4	3.5	2.3	21.6	24.8	6.2	10.8	56.0	73.1	18.1	24.4
Obese males	1.9	1.9	13.7	14.4	7.2	7.6	3.4	2.6	16.7	16.2	4.9	6.2	27.2	33.1	14.3	17.4
Lean follicular females	3.7	2.2	31.1	35.6	8.4	16.2	1.6	1.3	31.7	37.8	19.8	29.1	43.1	52.0	11.6	23.6
Lean luteal females	3.2	2.1	54.1	47.5	16.9	22.6	1.6	1.4	32.4	37.6	20.2	26.9	80.6	85.3	25.2	40.6
Obese females	3.6	2.5	18.7	22.0	5.2	8.8	1.1	1.0	12.0	12.8	10.9	12.8	62.0	49.6	17.2	19.8

Table A17: Comparing the effects of the *ex vivo* acute heart failure (AHF) model and trimetazidine (TMZ) treatment on all groups (n=6). The first RAHF column estimates the improvement as a percentage of the initial baseline (CAHF percentage x fold improvement in RAHF). Data represents the last ten minutes of each phase. RPP: rate pressure product, $(dp/dt)_{max}$: index of myocardial contraction velocity, CAHF: critical acute heart failure, RAHF: recovery acute heart failure.