

Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:

CHANDRAMOULI, CHANCHAL

Title:

Diabetic cardiomyopathy: sex-specific aspects of functional, structural and molecular remodelling

Date:

2017

Persistent Link:

<https://hdl.handle.net/11343/127453>

Terms and Conditions:

Terms and Conditions: Copyright in works deposited in Minerva Access is retained by the copyright owner. The work may not be altered without permission from the copyright owner. Readers may only download, print and save electronic copies of whole works for their own personal non-commercial use. Any use that exceeds these limits requires permission from the copyright owner. Attribution is essential when quoting or paraphrasing from these works.

Diabetic cardiomyopathy: sex-specific aspects of functional, structural and molecular remodelling

Chanchal Chandramouli
Bachelor of Biomedical Science (Hons)

Submitted in total fulfilment of the requirements of the degree of
Doctor of Philosophy

Mac 2017

Cardiac Phenomics Laboratory
Department of Physiology
Faculty of Medicine, Dentistry and Health Sciences
University of Melbourne



This Thesis is dedicated to
my ammamma,
Damayanthi Govindaraja.

Summary of contents

- Chapter 1** A review of the literature
- Chapter 2** General methodology
- Chapter 3** Diabetes-induced diastolic dysfunction is more marked in female mice, despite less pronounced hyperglycaemia
- Chapter 4** Differential responses to cardiac ischemia-reperfusion injury in T1D male and female mice
- Chapter 5** A signalling pathway survey in type-1 and type-2 diabetic myocardium of female and male mice
- Chapter 6** Type-1-diabetic cardiac functional and structural pathology is exacerbated by elevated cardiac AngII in male but not female mice
- Chapter 7** General discussion and conclusion
- Chapter 8** Appendix

Note: Chapters 3, 4, 5, and 6 (data chapters) are presented in the form of constructed manuscripts, which are ready for submission. For this reason some material from the Literature Review and Methodology chapters has been reiterated in the Introduction and Methods sections. References are included in each chapter as required for manuscript submission.

Abstract

Background: Clinical studies have revealed increased cardiovascular risk in diabetic patients, which is substantially elevated in women. Perplexingly, while there has been extensive experimental effort in characterising cardiac dysfunction in the progression of diabetic cardiomyopathy, studies investigating sex differences are limited. A mechanistic understanding of sexual dimorphism in diabetic cardiomyopathy remains to be achieved.

Aim: The aim of this Thesis was to examine female susceptibility to cardiac pathology in type 1 diabetes (T1D). In particular, this thesis focussed on examining cardiac responses to diabetes (functional and molecular) under basal conditions, during ischemia and with increased cardiac renin angiotensin system (RAS) signalling.

The four experimental questions addressed in this thesis are:

1. Are systemic and cardiac T1D phenotypes different between males and females? [Chapter 3]
2. Is there an accentuated female vulnerability to ischemia reperfusion injury in T1D? [Chapter 4]
3. Are there sex-specific changes in cell death, autophagy and metabolism associated with diabetes? [Chapter 5]
4. Does cardiac RAS upregulation interact with sex-specific cardiac responses in T1D? [Chapter 6]

Methods: A wide range of *in vivo*, *ex vivo* and molecular strategies were employed to characterise the role of sex differences in a streptozotocin (STZ)-induced T1D mouse model. Echocardiographic assessment was performed to examine T1D-induced functional and structural deficits *in vivo*. *Ex vivo* isolated heart perfusion analysis was used to characterise the role of sex differences in ischemia-reperfusion injury and recovery in T1D. The mechanistic basis of T1D-induced cardiac pathology was evaluated with various histological, biochemical and molecular techniques. Molecular findings from T1D models were also compared against changes from type 2 diabetic

(T2D) mouse models (lean and obese). Finally, the role of RAS in exacerbating the T1D phenotype was assessed using a cardiac-specific angiotensinogen overexpressing mouse model.

Results: The overall findings of this thesis are:

1. Although the extent of hyperglycaemia and increase in glycated haemoglobin (HbA1c) was less marked in female T1D in comparison to male T1D, diastolic dysfunction was evident in female T1D, but not in male T1D mice.
2. In males, diabetic hearts showed greater reperfusion recovery associated with reduced cardiac glycogen levels post-ischemia, suggesting better glycogen utilisation during ischemia, compared to male controls. In contrast, despite an earlier onset of ischemic contracture, the reperfusion recovery and glycogen levels were unchanged in female T1D hearts, compared to female control hearts.
3. *GABARAPL1*, a gene responsible for lysosomal breakdown of glycogen, was upregulated in T1D male hearts, whereas genes from conventional glycogen breakdown pathways (*glycogen phosphorylase* and *glycogen debranching enzyme*) were increased in female T1D. In addition, a pronounced increase in expression of genes from macro-autophagy pathway (protein bulk degradation) and apoptotic cell death pathway genes were observed in female T1D but not male T1D hearts. Interestingly, in lean and obese T2D mice, contrasting cardiac gene expression responses were observed in glycogen metabolic and macro-autophagy pathways.
4. With elevated cardiac AngII, T1D-induced cardiac functional and structural changes were exacerbated in males, but these changes were not apparent in females.

Conclusion: Collectively, the novel findings in this thesis have contributed new knowledge to the literature on sex-specific attributes of diabetic cardiomyopathy. This study is the first demonstration that a less pronounced hyperglycaemic response in T1D female mice is associated with more marked functional cardiac pathology. This

female vulnerability may be partially attributed to a preferential slower/inefficient processing of glycogen and heightened cell death pathology, evidenced from pronounced autophagic drive in female T1D mice. A sex-specific role for cardiac RAS in exacerbating the T1D phenotype has also been identified. The findings in this thesis support a case for sex-specific progression of diabetic cardiac pathology.

Declaration

This is to certify that:

- I. The Thesis comprises only my original work towards the PhD
- II. Due acknowledgement has been made in the text to all other material used
- III. The Thesis is fewer than 100, 000 words in length, exclusive of tables, bibliographies and appendices.

Chanchal Chandramouli

Chanchal

March 2017

Acknowledgements

I have had the immense privilege of being mentored by **Lea Delbridge** (Department of Physiology, University of Melbourne), **Kimberley Mellor** (Department of Physiology, University of Auckland) and **Rebecca Ritchie** (Heart Failure and Pharmacology Lab, Baker IDI Diabetic Institute) throughout my candidature. Thank you for your constant support and guidance throughout. To Lea and Kim, working closely with you both has been truly inspiring. Your “open office” policy, attention to detail and willingness to listen to my ideas encouraged me to meticulously analyse, challenge, interpret and re-interpret my data. Thank you all for giving me an opportunity to experience research in Australia and to work on this project.

I thank **Melissa Reichelt**, a former post-doc in the Cardiac Phenomics Lab (Department of Physiology, University of Melbourne) who began the STZ treatments for the first animal cohort and for performing the *ex vivo* cardiac functional experiments from which data were derived in components of Chapter 4 and 6. I would also like to acknowledge **Claire Curl** (Department of Physiology, University of Melbourne) for her assistance in performing the echocardiography experiments and for her staunch support during my candidature. Thanks to **David Stapleton**, a former academic in the Department of Physiology, University of Melbourne for training me in glycogen measurement using enzymatic assays. I would also like to express my gratitude to **Miles de Blasio** and **Helena Qin** (Heart Failure and Pharmacology Lab, Baker IDI) for training me in the HbA1c assays and for the useful insights on RT profiling experiments respectively.

I am also grateful for the generous financial support from **University of Melbourne** and the **Department of Physiology** in the form of a Melbourne International Research Scholarship and a Melbourne International Fee Remission Scholarship, without which, this project would not have been possible. I was also a fortunate recipient of the **Graduate Union** PhD scholarship 2014 (Graduate House), for which I would like to warmly thank the college. I would also like to acknowledge the expertise of Karin Hosking for providing assistance with proof reading service. The guidelines provided

by Australian Institute of Professional Editors (IPEd) for PhD thesis editing were adhered to.

To Cardiac Phenomics Lab members, **Brendan Ma, Upasna Varma, Laura Bienvenu, Antonia Raaijmakers, Gabriel Bernasochi, Johannes Janssens, Wendy Ip and Jim Bell**, thank you for all the experimental assistance during glucose tolerance tests and tissue collection. All your useful input and questions during lab meeting discussions helped me look at my data from different perspectives and further develop my project. It has been an absolute pleasure to work with a supportive and collegial team of scientists. Not forgetting the light-hearted cricket banter, coffee breaks, fructose stash and for their collaborative effort in helping me get over my Ornithophobia (partially)!

To my close friends, **Sant Kaur, Anbu Ganesh K, Pallavi Sinha, Saba Shaffakat, Sahil Khanna, Arunadevi Sathiaselan, Basma Shaker, Roberta Grant**, thank you for all the gastronomical adventures in Australia, your humour, care, love and friendship that got me through some of the more trying times of my PhD.

Finally, to my parents, **Chandramouli Annamalai and Chitra Govindaraja**, for their patience in proof reading, for their boundless care, love and stoic support at all times throughout my life.

Table of Contents

PREFACE

Dedication	ii
Summary of content	iii
Abstract	iv
Declaration	vii
Acknowledgements	viii
Table of Contents	x
List of Figures	xv
List of Tables	xvii
List of Abbreviations	xviii
Relevant Publications	xxii

CHAPTER 1: Review of the literature

1.1 Introduction.....	2
1.2 Clinical evidence of sex differences in diabetic cardiomyopathy	2
1.2A Physiologic and pathologic sex differences in systemic glucose regulation	3
1.2B Diabetic cardiomyopathy – sex contrast from symptoms to prognosis	4
1.2C Sex differences in cardiac pathology in diabetes	5
1.3 Experimental evidence of sex differences in diabetic cardiomyopathy	7
1.3A Systemic metabolic stress differences in males and females	7
1.3B Sex differences in diabetes-induced cardiac structural abnormalities	8
1.3C Sex differences in diabetes-induced cardiac functional abnormalities	12
1.3D Ischemia reperfusion - structural and functional injury	17
1.4 RAS/AngII involvement in diabetic cardiomyopathy	23
1.4A Upregulation of RAS in the diabetic heart	24
1.4B Possible signalling crosstalk between AngII and insulin signalling	25
1.5 Cardiac glycogen abnormalities in the diabetic heart	26
1.5A Altered myocardial metabolism and glycogen accumulation in diabetes	26

1.5B Glycogen flux pathways in the heart.....	27
1.5C Cardiac glycogen toxicity: Perspectives from glycogen storage diseases.....	30
1.6 Diabetes, sex and AngII in the heart – new questions	35
1.7 References.....	37

CHAPTER 2: General Methodology

2.1 Experimental animals.....	67
2.2 Induction of acute insulin deficiency by Streptozotocin in mice.....	67
2.3 The Angiotensinogen-Transgenic (AngII-Tg) mouse	67
2.4 Induction of chronic insulin deficiency by Streptozotocin in mice	68
2.5 Glucose Tolerance Testing	68
2.6 Echocardiography assessment of <i>in vivo</i> cardiac function	69
2.7 <i>Ex vivo</i> cardiac function and ischemia-reperfusion	69
2.8 Tissue, blood and plasma collection	70
2.9 Insulin ELISA measurement.....	70
2.10 Glycated haemoglobin measurement.....	71
2.11 Protein extraction through tissue homogenisation.....	71
2.12 Protein concentration determination	72
2.13 Enzymatic analysis of glycogen content.....	72
2.14 RNA extraction and quantification	72
2.15 Reverse transcription	73
2.16 RT profiling gene expression analysis.....	73
2.17 Picrosirius Red staining	76
2.18 Statistical analysis.....	76
2.18 References.....	77

CHAPTER 3: Diabetes-induced diastolic dysfunction is more marked in female mice, despite less pronounced hyperglycaemia

3.1 Introduction.....	80
3.2 Specific Methodology.....	82
3.3 Results	84
3.3A Female systemic phenotype is less pronounced than male in T1D	84

3.3B	Onset of STZ-induced systemic phenotype is delayed in females relative to males	84
3.3C	Female <i>in vivo</i> cardiac diastolic dysfunction is more severe than male..	85
3.3D	Mild systolic dysfunction is evident in female but not male mice	85
3.3E	Modest dilation and ventricular wall thinning with T1D in both sexes ..	86
3.3F	Increased myocardial collagen deposition with T1D in both sexes	86
3.3G	Sex differences in glucose and glycogen handling with T1D	87
3.4	Discussion.....	88
3.5	Conclusion and next questions.....	93
3.6	References.....	94
3.7	Tables and figures.....	101

CHAPTER 4: Differential responses to cardiac ischemia-reperfusion injury in type 1 diabetic male and female mice

4.1	Introduction.....	111
4.2	Specific Methodology.....	113
4.3	Results	115
4.3A	Basal left ventricular function in <i>ex vivo</i> hearts is not impaired by T1D.	115
4.3B	Despite earlier onset of contracture, ischemic injury is not different in female T1D mice.	116
4.3C	Pronounced functional rebound in male T1D but not in female T1D.....	116
4.4D	Reduced cardiac glycogen content in post-ischemic male T1D but not in female T1D.	117
4.4	Discussion.....	118
4.5	Conclusion and next questions.....	123
4.6	References.....	125
4.6	Tables and figures.....	130

CHAPTER 5: A signalling pathway survey in type-1 and type-2 diabetic myocardium of female and male mice

5.1	Introduction.....	137
5.2	Specific Methodology.....	140
5.3	Results	142

5.3A	Metabolic and cell death gene expression contrast in male and female T1D	142
5.3B	Gene expression profile differs between T1D and T2D, and lean and obese diabetic models	143
5.3C	Cardiac muscle fuel response is not different from skeletal muscle	145
5.4	Discussion.....	146
5.5	Conclusion and next questions.....	152
5.6	References.....	154
5.6	Tables and figures	162

CHAPTER 6: Type-1 diabetic cardiac functional and structural pathology is exacerbated by elevated cardiac AngII in male but not female mice

6.1	Introduction.....	167
6.2	Specific Methodology.....	169
6.3	Results	170
6.3A	<i>In vivo</i> diastolic dysfunction is evident in male and female T1D mice with elevated cardiac AngII.	170
6.3B	<i>In vivo</i> systolic dysfunction is not evident in male and female T1D mice with elevated cardiac AngII.....	171
6.3C	Left ventricular dilation and wall thinning is evident in male but not female T1D mice with elevated cardiac AngII.....	171
6.3D	<i>Ex vivo</i> female ischemic injury in T1D mice with elevated cardiac AngII is less severe than in male mice.....	172
6.3E	No effect of T1D on contractile recovery in isolated hearts post-ischemia in male and female mice with elevated cardiac AngII.	172
6.4	Discussion.....	174
6.5	Conclusion	177
6.6	References.....	178
6.6	Tables and figures	182

CHAPTER 7: General discussion and conclusion

7.1	Overview.....	191
-----	---------------	-----

7.2	Evidence of distinct systemic and cardiac diabetic pathology in both males and females.....	193
7.3	Female vulnerability in ischemia reperfusion linked with diminished glycogen utilisation.....	194
7.4	Differential expression of glycogen handling, autophagy and apoptosis genes in diabetic male and female hearts	195
7.5	T1D-induced cardiac functional and structural detriments are exacerbated by elevated cardiac AngII in male but not female mice	197
7.6	New research questions.....	198
7.7	Overall conclusions.....	199
7.8	References.....	200

CHAPTER 8: Appendix

Appendix 1:	Glycogen assay optimisation	207
Appendix 2:	RNA quantitation and quality control	220
Appendix 3:	Published papers and submitted abstracts	224

List of Figures

Figure 1.1.	Glycogen structure and breakdown in the cytosol	32
Figure 1.2.	Distinct cardiomyocyte immunohistologic localisation of glycogen autophagic trafficking cargo in vitro	33
Figure 1.3.	Hypothesis for interactions between insulin, AngII and estrogen signalling pathways	34
Figure 3.1.	Systemic characteristics of T1D in male and female mice	103
Figure 3.2.	Acute systemic response to insulin withdrawal in male and female mice	104
Figure 3.3.	Diastolic dysfunction is more severe in T1D female mice	105
Figure 3.4.	Mild systolic dysfunction is T1D female mice	106
Figure 3.5.	Cardiac morphological characteristics of T1D mice.	107
Figure 3.6.	Cardiac fibrotic infiltration in T1D in male and female mice	108
Figure 3.7.	Expression of glucose-related metabolism genes and glycogen content in T1D male and female mouse hearts	109
Figure 4.1.	No effect of T1D on basal left ventricular function in <i>ex vivo</i> male and female mouse hearts	131
Figure 4.2.	Sex differences in ischemic injury in T1D mice.....	132
Figure 4.3.	Sex differences in the recovery of contractile function post 20min ischemia in T1D mice	133
Figure 4.4.	Left ventricular function at 30mins reperfusion of <i>ex vivo</i> T1D male and female mice hearts.....	134
Figure 4.5.	Differential glycogen utilisation in male and female T1D mouse hearts post ischemia-reperfusion	135
Figure 5.1.	Sex differences in energy utilisation and cell death pathways in T1D mice	163
Figure 5.2.	Energy utilisation and cell death pathway gene expression responses in T1D and T2D male mice	164
Figure 5.3.	Energy utilisation and cell death pathway in cardiac vs. skeletal muscle of T1D male mice.....	165
Figure 6.1.	Diastolic dysfunction is evident in male and female T1D mice with elevated cardiac AngII	185
Figure 6.2.	Systolic dysfunction is not evident in male and female T1D mice with elevated cardiac AngII	186

Figure 6.3.	Left ventricular dilation and wall thinning is evident in male but not female T1D mice with elevated cardiac AngII	187
Figure 6.4.	Female ischemic injury in T1D mice with elevated cardiac AngII is less severe than in male mice	188
Figure 6.5.	No effect of T1D on contractile recovery post-ischemia in male and female mice with elevated cardiac AngII.....	189
Figure 7.1.	Summary of key thesis findings on sex differences in functional, structural and molecular remodelling in diabetic cardiomyopathy	199
Figure 8.1.	Original glycogen assay protocol.....	208
Figure 8.2.	Optimised final glycogen assay protocol for cardiac homogenates.....	219

List of Tables

Table 1.1.	Cardiac morphology and functional characteristics in experimental models of T1D and T2D.....	21
Table 1.2.	Sex-specific features of cardiac responses observed in experimental models of T1D and T2D.....	22
Table 2.1.	List of genes included in the RT ² Profiler PCR Array plate.....	75
Table 3.1	Echocardiographic function characteristics of T1D mice.....	101
Table 3.2.	Cardiac morphological characteristics of T1D male and female mice	102
Table 4.1.	Systemic and cardiac morphologic characteristics of T1D male and female mice	130
Table 5.1	Systemic characteristics of diabetic rodent models vs. controls.	162
Table 6.1	Systemic and cardiac morphologic characteristics of T1D male and female AngII-Tg mice.....	182
Table 6.2	Echocardiographic function characteristics of T1D males and female mice	183
Table 6.3	Basal <i>ex vivo</i> cardiac function in T1D AngII-Tg mice	184
Table 7.1.	Summary of gene expression changes with T1D in both sexes.....	196
Table 8.1.	Analysis of RT ² RNA QC PCR Array Results	221
Table 8.2.	Nanodrop RNA concentration and purity results.....	222

List of Abbreviations

A	late diastolic filling
A'	late diastolic mitral annular velocity
abs	absorbance
ACE	angiotensin converting enzyme
ACE <i>i</i>	angiotensin converting enzyme inhibitor
Acetyl-CoA	coenzyme A
AEC	animal ethics committee
AIM	Atg-8 interacting motif
Akt/PKB	protein kinase B
AMI	acute myocardial infarction
AMPK	AMP-activated kinase
AngII	angiotensin II
AngII-Tg	angiotensin II transgenic
ANOVA	analysis of variance
AOGN	angiotensinogen
ARBs	angiotensin receptor blockers
ArKO	aromatase-knockout
AT ₁	angiotensin II type 1 receptor
AT ₁ -KO	angiotensin receptor type 1 knockout
AT ₂	angiotensin II type 2 receptor
Atg7	Autophagy-related protein 7
Atg8	Autophagy-related protein 8
AUC	area under the curve
Bax	Bcl2-associated X protein
Bcl2	B-cell leukemia/lymphoma 2
bpm	beats per minute
Ca ²⁺	calcium ion
cDNA	deoxyribonucleic acid synthesised from messenger ribonucleic acid
C _t	threshold cycle
Ctrl	control
CVD	cardiovascular disease

CWI	cardiac weight index
DAPI	4',6-diamidino-2-phenylindole
db/db	mice with homozygous mutation in leptin receptor
Dec T	deceleration time
DevP	developed pressure
DNA	deoxyribonucleic acid
$dP/dt_{\max}$	maximum rate of contraction
$dP/dt_{\min}$	maximum rate of relaxation
DWT	diastolic wall thickness
E	early diastolic filling
E'	early diastolic mitral annular velocity
E'/A'	mitral peak early to late annular velocity ratio
E/A	mitral peak early to late filling velocity ratio
E/E'	mitral peak early filling velocity to early diastolic mitral annular velocity ratio
ECG	electrocardiogram
EDP	end diastolic pressure
ELISA	enzyme-linked immunosorbent assay
ERT	estrogen replacement therapy
ER α	estrogen receptor- α
F	female
FDG	2-deoxy-2-(^{18}F)fluoro-D-glucose
G6P	glucose-6-phosphate
Gaa	lysosomal alpha-glucosidase α
GABA $_A$	gamma-Aminobutyric acid
GABARAPL1	GABA Type A Receptor Associated Protein Like 1
GDE	glycogen debranching enzyme
gDNA	genomic deoxyribonucleic acid
GLUT4	glucose transporter type 4
GSD	glycogen storage disorder
GTT	glucose tolerance test
H $_2$ O $_2$	hydrogen peroxidase
Hb	haemoglobin

HbA1c	glycated haemoglobin
HFpEF	heart failure with preserved ejection fraction
HFrfEF	heart failure with reduced ejection fraction
IRS1	insulin receptor substrate 1
IVRT	interventricular relaxation time
IVSd	interventricular septal end diastole
IVSs	interventricular septal end systole
JNK	c-Jun N-terminal kinase
LAMP 2	lysosome-associated membrane protein 2
LC3	microtubule-associated protein 1 light chain 3
LSD	least significant difference
LV	left ventricle
LVIDd	left ventricular internal diameter end diastole
LVIDs	left ventricular internal diameter end systole
LVPWd	left ventricular posterior wall end diastole
LVPWs	left ventricular posterior wall end systole
M	male
MAPK	mitogen-activated protein kinase
MHC	myosin heavy chain
mins	minutes
MR	mineralocorticoid receptor
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
N ₂	nitrogen
NaAc	sodium acetate
NRT	no reverse transcription control
NTC	no template control
ob/ob	mice homozygous for the obese spontaneous mutation
P13K	phosphoinositide 3-kinase
PCR	polymerase chain reaction
PET/CT	positron emission tomography-computed tomography
PGO	peroxidase glucose oxidase
PHT	pressure half time

PI3K α	class 1A PI3K
PPC	positive PCR control
PW	pulse-wave
PWT	posterior wall thickness
QC	quality control
RAS	renin-angiotensin system
RCF	relative centrifugal force
RNA	ribonucleic acid
ROS	reactive oxygen species
RPM	revolutions per minute
RT	reverse transcription
RTC	reverse transcription control
RWT	relative wall thickness
SEM	standard error of mean
STBD1	starch-binding domain-containing protein 1
STD	standard
STZ	streptozotocin
T1D	type 1 diabetes
T2D	type 2 diabetes
Tg	transgenic
TGF β	transforming growth factor β
vol	volume

Relevant Publications

Thesis-related publications

1. **Chandramouli C**, Varma U, Stevens E, Stapleton D, Mellor KM, Delbridge LMD. Myocardial glycogen dynamics: new perspectives on disease mechanisms. *Clinical and Experimental Pharmacology and Physiology*. 2015 Apr;42(4):415-25.
2. Reichelt ME, Mellor KM, Bell JR, **Chandramouli C**, Headrick JP, Delbridge LMD. Sex, sex steroids and diabetic cardiomyopathy: making the case for experimental focus. *Am J Physiol Heart Circ Physiol*. 2013 Sep 15;305(6):H779-92.
3. **Chandramouli C***, Reichelt ME*, Varma U, Curl CL, Ritchie RH, Mellor KM[#], Delbridge LMD[#]. Diabetes-induced diastolic dysfunction is more marked in female mice, despite less pronounced hyperglycaemia. *Biol Sex Differ*. [In preparation – Chapter 3].
4. **Chandramouli C**, Reichelt ME, Ma, B, Qin CX, De Blasio M, Ritchie RH, Mellor KM[#], Delbridge LMD[#]. Distinctive cardiomyocyte trophic, viability and energy regulatory signalling pathways in type-1 and type-2 diabetic myocardium of female and male mice. *Am J Physiol Heart Circ Physiol*. [In preparation – Chapter 5].
5. **Chandramouli C***, Reichelt ME*, Bienvenu LA, Curl CL, Ritchie RH, Mellor KM[#], Delbridge LMD. [#] Type-1-diabetic cardiac functional and structural pathology is exacerbated by elevated cardiac angiotensin II in male but not female mice. *Cardiovasc Diabetol*. [In preparation – Chapters 4 and 6].

Other associated publications

6. Mellor KM, Curl CL, **Chandramouli C**, Pedrazzini T, Wendt IR, Delbridge LMD. Aging-related cardiomyocyte functional decline is sex- and angiotensinII-dependent. *Age (Dordr)* 2014 Jun; 36(3):9630.

7. Ip TK, **Chandramouli C**, Smith JA, McLennan PL, Pepe Salvatore, Delbridge LMD. A Small Cohort Omega-3 PUFA Supplement Study: Implications of Stratifying According to Lipid Membrane Incorporation in Cardiac Surgical Patients. *Am J Physiol Heart Circ Physiol*. [In press]
8. Kennedy TL, Swiderski K...Curl CL, **Chandramouli C**, Febbraio MA, Delbridge LMD, Koopman R, Lynch GS. BGP-15 improves aspects of the dystrophic pathology in mdx and dko mice with differing efficacies in heart and skeletal muscle. *Am J Path*. [In press]
9. Curl CL, Danes VR, Bell JR, Raaijmakers AJA, Ip WTK, **Chandramouli C**, Harding TW, Mellor KM, Crossman DJ, Kalman JM, Soeller C, Harrap SB, Delbridge, LMD. Cardiomyocyte Ca²⁺-handling and arrhythmogenic pathophysiology is distinctive in heart failure with preserved ejection fraction (HFpEF). *Circ Res*. [submitted]

Conference Abstracts

1. Reichelt ME, **Chandramouli C**, Chan, Z, Mellor KM, Ritchie RH, Curl CL, Delbridge LMD. Elevated cardiac AngII leads to structural and mechanical dysfunction in diabetic normotensive mice. Proceedings from the Australian Physiological Society Meeting, Geelong, Dec 2013, Poster presentation.
2. **Chandramouli C**, Reichelt ME, Mellor KM, Ritchie RH, Curl CL, Delbridge LMD. Elevated cardiac AngII influences diabetic myocardial pathology differently in males and females. Proceedings from the Australian Health and Medical Research Congress, Nov 2014. Poster presentation.
3. **Chandramouli C**, Reichelt ME, Mellor KM, Ritchie RH, Curl CL, Delbridge LMD. Sex differences in AngII-mediated exacerbation of diabetic myocardial function. Proceedings from the International Society of Heart Research (Australasian Section) Meeting, Melbourne, Aug 2015.
4. **Chandramouli C**, Reichelt ME, Mellor KM, Ritchie RH, Curl CL, Delbridge LMD. Diabetic myocardial pathology is more severe in female than male mice.

Proceedings from National Heart Association of Malaysia Meeting, Apr 2016,
Poster presentation [submitted]

5. Curl CL, Raaijmakers AJA, **Chandramouli C**, Harding TW, Harrap SB, Delbridge LMD. An unexpected dichotomy in whole heart and CM function in the HHR rat: A model of hypertrophy in the absence of hypertension. Proceedings from High Blood Pressure Research Council of Australia Meeting, Nov 2013. Oral presentation (presented by Curl CL)
6. Brock CJ, Swiderski K, Murphy KT, **Chandramouli C**, Curl CL, Delbridge LMD and Lynch GS. Characterisation of cardiac cachexia in tumour-bearing mice. Proceedings from the Gage Muscle Conference, Canberra, Australia, Apr 2014. Poster presentation (presented by Brock CJ)

CHAPTER 1

Review of the literature

Manuscripts derived from this chapter:

Reichelt ME, Mellor KM, Bell JR, **Chandramouli C**, Headrick JP, and Delbridge LM. Sex, sex steroids, and diabetic cardiomyopathy: Making the case for experimental focus. *American Journal of Physiology: Heart and Circulatory Physiology* 2013 305: H779-792.

Chandramouli C, Varma U, Stevens EM, Xiao RP, Stapleton DI, Mellor KM, and Delbridge LM. Myocardial glycogen dynamics: New perspectives on disease mechanisms. *Clinical and Experimental Pharmacology & Physiology* 2015 42: 415-425.

1.1 Introduction

Diabetes is a disease of epidemic proportions and cardiovascular complications are the leading cause of diabetic mortality and morbidity worldwide (Boudina *et al.*, 2007). While the occurrence of type 2 diabetes (T2D) is considerably higher than type 1 diabetes (T1D), the incidence of both is rising steadily. In Australia, almost three-quarters of deaths among diabetic populations are attributable to cardiovascular disease, and of all cardiovascular deaths, 17.4% are due to diabetes (NHFA, 2007).

Diastolic dysfunction (preceding systolic abnormality) is an early sign of diabetic cardiomyopathy (Boyer *et al.*, 2004; Zabalgaitia *et al.*, 2001), linked with increased chamber stiffness and abnormal filling (Galderisi, 2006; Liu *et al.*, 2001; van Heerebeek *et al.*, 2008). Several etiological factors have been proposed to explain the development of diabetic cardiomyopathy. This includes increased diffuse interstitial collagen deposition, with perimysial and perivascular collagen accumulation (Regan *et al.*, 1977; Shimizu *et al.*, 1993), which may contribute to diastolic stiffness (Chowdhry *et al.*, 2007; Frustaci *et al.*, 2000). Metabolic changes include the accumulating evidence of impaired glucose signalling (An, 2006) and glycogen accumulation, which has been reported in human (Sakakibara *et al.*, 2011; Warren, 1930) and rodent diabetic models (Alfarano *et al.*, 2011; Lajoie *et al.*, 2004; Shearer *et al.*, 2011). The Framingham Heart Study identified increased cardiovascular risk in the diabetic population, with particularly exacerbated risk in women (Kannel, 1979). Growing clinical evidence has subsequently highlighted a steady increase in detrimental consequences of diabetic cardiomyopathy with diabetes and females are particularly susceptible. The subsequent sections in this review focus on the current literature relating to clinical and experimental evidence of sex differences in diabetic cardiomyopathy, and the involvement of the renin-angiotensin system (RAS) and glycogen pathology in diabetic cardiomyopathy, while highlighting the necessary knowledge deficit which needs to be addressed.

1.2 Clinical evidence of sex differences in diabetic cardiomyopathy

Physiological sex differences in the insulin regulation of glucose levels in men and women have been reported. There is a general recognition of sex differences in cardiovascular morbidity and mortality, with risk reduced among non-diabetic women (Editorial, 2011; Hayes *et al.*, 2011b; Vaccarino *et al.*, 2011). Accumulating evidence

reveals that diabetes pathology may abrogate this “protection” and make women more vulnerable to CVD compared to men (Barrett-Connor *et al.*, 1991; Folsom *et al.*, 1997; Heyden *et al.*, 1980; Kannel *et al.*, 1979; Natarajan *et al.*, 2003; Simons *et al.*, 1996).

For both men and women, T1D is associated with increased cardiovascular risk, with cardiovascular disease being the leading cause of death from 30 years of age onwards (Laing *et al.*, 1999). At age 55, cumulative mortality in T1D men and women is ~35% (compared to 4-8% in non-diabetics) (Krolewski *et al.*, 1987). Development of cardiac hypertrophy occurs in early stages of T1D (El Dayem *et al.*, 2012), and diastolic dysfunction is the most common echocardiographic manifestation of T1D clinically (Palmieri *et al.*, 2008; Schannwell *et al.*, 2002).

The incidence of T2D is considerably higher than T1D. For men and women combined, approximately 65% of T2D deaths are attributed to cardiovascular disease, involving increased coronary heart disease related risk in particular (Grundy *et al.*, 1999). The prevalence of subclinical left ventricular dysfunction in otherwise healthy T2D men and women is significant (Fang *et al.*, 2005). With disease progression, increased left ventricle mass, wall thickness and in some instances chamber dilation are reported in T2D populations (Devereux *et al.*, 2000). A component of this cardiac structural modelling may be attributed to coincident obesity, and not necessarily diabetes *per se* (Galvan *et al.*, 2000; Kuperstein *et al.*, 2001).

1.2A Physiologic and pathologic sex differences in systemic glucose regulation

Several clinical studies have reported basal sex differences in systemic glucose regulation. While similar elevations in blood glucose response to dextrose infusion were observed, systemic insulin was higher in females, suggestive of lower basal insulin sensitivity in females (Flanagan *et al.*, 2006). Insulin-stimulated disposal of radiolabeled glucose may be reduced in young adult females compared to age-matched males (Basu *et al.*, 2006). Administration of estrogen/progesterone through oral contraceptive use has been associated with a 40% reduction in insulin sensitivity (Perseghin *et al.*, 2001). In contrast, aging in women (with associated reductions in gonadal estrogen production) was associated with a more marked reduction in insulin sensitivity than that observed in men (Borissova *et al.*, 2005). Thus, though a physiological sex difference has been demonstrated, the directional influence of estrogen on insulin sensitivity is still ambiguous in the clinical literature.

Despite these basal sex differences in glucose regulation, elevated fasting glucose is used as the primary diabetic diagnostic criterion as recommended by the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (ECD/CDM, 2003). While elevated fasting glucose is common in diabetic males (Glumer *et al.*, 2003; Sicree *et al.*, 2008), glucose dysregulation in females is more frequently characterised by a prolonged duration of hyperglycaemia following acute glucose loading (Dotevall *et al.*, 2007; Glumer *et al.*, 2003; Sicree *et al.*, 2008; Williams *et al.*, 2003). Prolonged high estrogen levels in the luteal phase of the menstrual cycle have been associated with decreased insulin sensitivity in some T1D women (Widom *et al.*, 1992). It is thus suggested that the use of fasting blood glucose leads to under-diagnosis of diabetic females and underrepresentation in clinical trials (Pomerleau *et al.*, 1999). Collectively, these observations support fundamental differences in insulin-dependent glucose homeostasis in women vs. men in both normoglycemic and hyperglycaemic settings.

1.2B Diabetic cardiomyopathy – sex contrast from symptoms to prognosis

Three decades ago, the Framingham Heart Study first identified a 5-fold increase in risk of heart failure in diabetic females compared to a 2-fold increase in risk in males (Kannel *et al.*, 1979). Several large studies support this notion of greater relative risk of heart disease (or fatal cardiovascular events) with diabetes in females vs. males (Barrett-Connor *et al.*, 1991; Folsom *et al.*, 1997; Heyden *et al.*, 1980; Natarajan *et al.*, 2003; Simons *et al.*, 1996). In general, cardiovascular disease presentation in women is delayed by about 10 years in comparison with men, but this relative “*protective-delay*” is abrogated in diabetic women (Norhammar *et al.*, 2013). For women the post-menopause increase in cardiac risk occurs in parallel with an increase in the detected incidence of insulin resistance and diabetes (Kannel *et al.*, 1979) where both coronary and myocardial pathology involvement could be implicated. Whether menopause *per se* exacerbates the risk to a greater extent in diabetics than non-diabetics is not fully understood. Diabetic females are also characterised by higher risk of acute myocardial infarction (AMI) than their male diabetic counterparts (Kappert *et al.*, 2012; Lam *et al.*, 2012). Sex differences in cardiac presentation symptoms and detection of acute myocardial events are also evident. Men commonly describe acute chest/left arm pain or diaphoresis, while women report nausea, jaw pain and lethargy (Arslanian-Engoren, 2006). High fasting blood glucose levels are more event-predictive for men than women

(Takada *et al.*, 2012; Yang *et al.*, 2011), consistent with the observation that lower levels of hyperglycaemia may be of more pathologic significance in females as witnessed in Framingham offspring participants (Levitzy, 2008). Therefore, diabetic women frequently experience an asymptomatic or silent myocardial infarction with a slight elevation in blood glucose.

Worsened post-infarction outcomes for diabetic women have been documented in large-scale clinical studies conducted worldwide, across different age groups (Crowley *et al.*, 2003; Fraser *et al.*, 1992; Hayes *et al.*, 2011a; Lundberg *et al.*, 1997; Norhammar *et al.*, 2008; Seeman *et al.*, 1993). In some studies, overall sex differences have not been apparent, yet thorough sub-group analyses have revealed otherwise. A retrospective analysis revealed a 52% increase in mortality rate in young diabetic women (vs. men) evident during 5-year follow ups post AMI (Ouhoumane *et al.*, 2009). In another study, while the relative risk of CVD-related death was greater in diabetic males than females, a greater risk in females was apparent when participants with previous events were excluded (Kleinman *et al.*, 1988).

Heart failure manifests either with reduced ejection fraction (HFrEF) or with preserved ejection fraction (HFpEF) (Gladden *et al.*, 2014). Diabetic cardiomyopathy, as a predominantly diastolic dysfunction, has been described as a feature of HFpEF. Interestingly, HFpEF occurs more commonly in older individuals and women. Current treatment options with beta-blockers etc. cater towards management of HFrEF. No specific proven medical therapies are available for the treatment for HFpEF (Boonman-de Winter *et al.*, 2016; Gladden *et al.*, 2014; van Heerebeek *et al.*, 2016).

Therefore, distinct sex differences in clinical risk, prevalence, symptom presentation, detection and prognosis of cardiovascular diseases are evident. Further cardiac-specific structural, functional and metabolic changes in the diabetic state are investigated in the next section, with particular focus on available the literature on sex differences in T1D and T2D.

1.2C Sex differences in cardiac pathology in diabetes

Some cardiac- and sex-specific characteristics are available both in clinical T1D and T2D settings. Ischemic heart disease in young T1D women has been associated with exceptionally high mortality (Laing *et al.*, 2003; Secrest *et al.*, 2010), although this dimorphism may not be as apparent in middle-aged and older cohorts (Laing *et al.*,

1999). A study consisting of T1D children and adolescents identified significant increases in left ventricular wall dimensions and diastolic dysfunction in females but not in males, with higher glycosylated haemoglobin in female diabetics suggesting greater dysregulation of glucose handling in females (Suys *et al.*, 2004). Early occurrence of diastolic dysfunction in young female T1Ds is especially notable. As these young patients have no confounding hemodynamic and atherosclerotic complications the argument for a primary myocardial defect (with female-specific exacerbation) with T1D is compelling.

For T2D women, and for women with clinical indication of glucose intolerance, angina has been associated with increased risk of coronary heart disease mortality (Carpiuc *et al.*, 2010). One study estimated a substantial sex differential in the T2D related hazard ratio for major coronary heart disease even after adjustment for other risk factors to be 2.8 in males compared with 9.5 in females (Juutilainen *et al.*, 2004). As a cohort, T2D females exhibit greater symptomology though less obstructive coronary artery disease compared to diabetic males (Tamis-Holland *et al.*, 2011). Post-infarct prognosis for T2D women is poorer than for men, but increased mortality and morbidity may also be related to age differences, not simply attributable to sex (Venskutonyte *et al.*, 2010). Consistent with findings from studies of pooled T1D/T2D populations, T2D females are more likely to have other cardiovascular risk factors (dyslipidaemia, obesity, hypertension) and the impact of these on the incidence of cardiovascular disease is more severe in women (Juutilainen *et al.*, 2004). T2D women are reportedly less likely to receive treatment than men in comparable risk-factor settings (Chou *et al.*, 2007; Huxley *et al.*, 2006). This may have important outcome consequences, and confound the evaluation of underlying sex-specific differences.

In overview, clinical and population studies suggest diabetes induces significant and selective risk for women, particularly in relation to the occurrence and consequences of coronary artery disease, post infarct outcomes, and progression to heart failure. There is some evidence, from both T1D and T2D settings, that younger women are more adversely affected by diabetes, exhibiting cardiac symptomatic jeopardy, even when the level of hyperglycaemia may be lower than in men. This female vulnerability may partially reflect underlying sex differences in systemic glucose handling, and a lower pathological threshold for plasma glucose elevation. Estrogen may have a role in long-

term modelling and acute modulation of cardiac structure and function, although this is yet to be explicitly investigated.

1.3 Experimental evidence of sex differences in diabetic cardiomyopathy

Given the accumulating evidence of sex differences in the clinical diabetic context, this section therefore examines the available literature on experimental diabetic models to dissect the underlying cardiac-specific and sex-specific pathology. Experimental studies have enabled detailed and mechanistic characterisation of diabetes-associated structural and functional alterations to the myocardium. These studies outlined below (and tabulated, Table 1.1) involve either distinct male (most commonly) or female cohorts, or consist of pooled male/female data. Investigations specifically addressing sex-specificity of diabetic cardiopathology are small in number and have been highlighted in Table 1.2.

1.3A Systemic metabolic stress differences in males and females

A number of experimental studies have described sex differences in insulin responses and systemic glucose handling, somewhat consistent with clinical findings. Healthy female rat hearts appear less sensitive to insulin than male hearts (Vital *et al.*, 2006), and global deletion of the estrogen receptor (ER α) is associated with systemic hyperglycaemia and glucose intolerance (Ruegg *et al.*, 2011). Interestingly, ovariectomy *per se* did not modify insulin resistance or blood glucose levels (Galipeau *et al.*, 2002a). Insulin and estrogen both exert regulatory influences on the Akt/PI3K signalling pathway (depicted in Figure 1.3), and estrogen receptor activation is known to modulate insulin signalling (Gao *et al.*, 2002; Patten *et al.*, 2004). Given sex-specific differences in the systemic milieu of glucose and insulin modulation in normoglycemic settings, sex disparity in diabetic responses might be anticipated. An interaction between insulin and estrogen signalling has the potential to modulate numerous downstream cardiac structural and functional characteristics associated with diabetes.

In T1D, sex differences have been described in the development of hyperglycaemia in some animal models. The widely used streptozotocin (STZ) model of T1D involves selective destruction of pancreatic β -cells. As a result, rodents experience insulin deficiency, hyperglycaemia and polydipsia, characteristic of human T1D (Furman, 2015). In mice, the usage of a multiple low-dose (40-55mg/kg x 5 injections) STZ

approach has been shown to partially destroy pancreatic islets and trigger an inflammatory process that causes further loss of β -cell activity, resulting in a chronic T1D phenotype within 7 weeks post injections (Furman, 2015). The extent of hyperglycaemia in response to STZ treatment is reported to be similar in male and female rats (Ding *et al.*, 2006), while STZ combined with nicotinamide accentuates development of hyperglycaemia in female rats (Vital *et al.*, 2006). Higher glucose levels were attributed to greater β -cell destruction (Vital *et al.*, 2006), which may be related to anti-apoptotic β -cell sparing effects of testosterone (Palomar-Morales *et al.*, 2010). STZ-induced hyperglycaemia has been shown to be more or less marked in females, or equivalent in males and females depending on the strain studied (Ceylan-Isik *et al.*, 2006a; b; Matsumoto *et al.*, 2008; Nuno *et al.*, 2010). Notably, hyperglycaemia in the T1D experimental setting has been observed to be less prominent in females of the widely employed C57Bl6 strain (Nuno *et al.*, 2010).

In the context of T2D, occurrence of systemic insulin resistance is reportedly less marked in female rodents exposed to elevated dietary fructose (Galipeau *et al.*, 2002a), elevated dietary sucrose (Horton *et al.*, 1997), or within a hyperinsulinaemic setting (Galipeau *et al.*, 2002b). This more modest female insulin resistance/hyperglycaemia is likely sex steroid influenced, as ovariectomy abrogates protection against hyperinsulinaemia (Galipeau *et al.*, 2002a). In a T2D genetic model (Goto-Kakizaki), although plasma glucose was not sex-dependent, disturbances in plasma lipids were observed only in females (Desrois *et al.*, 2004).

Thus, ambiguities relating to systemic diabetic clinical characterisation of women (relative to men) are observed to some degree in experimental settings. Experimental manipulations may not necessarily produce equivalent systemic phenotypes in females vs. males, and presumption that all individuals within a treatment cohort possess similar disease states (as gauged by glycaemic state or insulin resistance) is questionable. Beyond questions of sex differences in systemic insulin sensitivity, sex differences in the structural and functional cardiac implications of the diabetic state have received minimal experimental attention.

1.3B Sex differences in diabetes-induced cardiac structural abnormalities

As observed clinically, increased cardiac fibrosis and collagen deposition is a characteristic of experimental T1D (Kato *et al.*, 2011; Li *et al.*, 2011; Meloni *et al.*,

2012) and T2D (Daniels *et al.*, 2011; Mellor *et al.*, 2011; Zhou *et al.*, 2011) models. Work in the STZ T1D model shows that stimulation of TGF β in a hyperglycaemic context promotes fibroblast collagen production, and suggests heightened angiotensin II signalling is frequently involved (Benazzoug *et al.*, 1998; Connelly *et al.*, 2007a; Singh *et al.*, 2008). Acting through the AT-1 receptor, angiotensin decreases matrix metalloprotein-2 activity in diabetic (STZ) mice, suppressing collagen degradation and promoting cardiac fibrosis (Westermann *et al.*, 2007). In the T2D experimental setting, upregulated TGF β -receptor signalling has also been observed with collagen deposition a precursor to hyperglycaemia (Mizushige *et al.*, 2000). Increased apoptotic cardiomyocyte loss and increased oxidative stress are evident with fibrotic infiltration in both T1D and T2D (Barouch *et al.*, 2006; Fiordaliso *et al.*, 2000). While the remodelling process leading to diastolic dysfunction has been extensively studied, characterisation of sex-specific myocardial impacts of diabetes awaits future investigation.

Cardiac hypertrophy is not always evident in experimental models of diabetes. Rodent models of T1D are typically untreated, resulting in severe hyperglycaemia and a reduction in or failure to gain bodyweight. In male T1D this can result in *higher* heart weight to body weight ratios (Hoit *et al.*, 1999; Hu *et al.*, 2011; Nawata *et al.*, 2002; Ooie *et al.*, 2003; Raimondi *et al.*, 2004; Ren *et al.*, 2000; Silva *et al.*, 2011; Takahashi *et al.*, 2003; Vadlamudi *et al.*, 1982; Wold *et al.*, 2001; Xie *et al.*, 2011), independent of heart weight changes, or, no differences are observed in heart weight or normalised heart weight. In females, STZ-induced changes in heart weight indices were increased (Vadlamudi *et al.*, 1982) or unchanged (Yu *et al.*, 2007), with persisting increase in heart weight observed even after normalisation (Ceylan-Isik *et al.*, 2006b). In male T1D rodents, ventricular cardiomyocyte size is reported to be larger (Huynh *et al.*, 2010; Khong *et al.*, 2011; Li *et al.*, 2011; Ritchie *et al.*, 2012; Ti *et al.*, 2011; Wold *et al.*, 2006) or unchanged (Curl *et al.*, 2001; Hofmann *et al.*, 1995; Kotsanas *et al.*, 2000; Raimondi *et al.*, 2004; Roe *et al.*, 2011; Shao *et al.*, 2011; Shao *et al.*, 2010; Wei *et al.*, 2009) or less commonly reduced (Silva *et al.*, 2011), and left ventricular wall thickness is reported to be reduced (Meloni *et al.*, 2012; Roe *et al.*, 2011) or unchanged (Ho *et al.*, 2006; Strunz *et al.*, 2011). In studies of females, cardiomyocyte size was unchanged (Ceylan-Isik *et al.*, 2006b; Hofmann *et al.*, 1995; Howarth *et al.*, 2005). One study

identified decreased cardiomyocyte size, though it is not clear which sex was studied (Silva *et al.*, 2011).

In T2D animal models the situation regarding cardiac hypertrophy appears even more complex, sometimes confounded by animal obesity rather than body weight loss. Tibial length is sometimes preferred to body weight for normalisation purposes, but with genetic models of diabetes this may also be altered relative to control (Daniels *et al.*, 2011). Many studies in males report *no change* in heart weight in T2D models, including Zucker rats (Howarth *et al.*, 2012; Oakes *et al.*, 2006; Wang *et al.*, 2005), leptin-receptor deficient (*db/db*) mice (Hafstad *et al.*, 2006; Panagia *et al.*, 2007; Park *et al.*, 2009), leptin deficient (*ob/ob*) mice (Mascareno *et al.*, 2009), fructose-fed animals (Chang *et al.*, 2007; Chess *et al.*, 2007; Mellor *et al.*, 2011), sucrose-supplemented rats (Vasanji *et al.*, 2006), Goto-Kakizaki diabetic rats (Howarth *et al.*, 2008), high-fat fed rats (Ouwens *et al.*, 2007) and Otsuka Long-Evans Tokushima Fatty rats (Kim *et al.*, 2003). When normalised to body weight, this lack of effect in cardiac size persists in the Zucker rats (Baynes *et al.*, 2009; Howarth *et al.*, 2012; Wang *et al.*, 2004), fructose-fed animals (Chang *et al.*, 2007; Chess *et al.*, 2007; Mellor *et al.*, 2011) and in Otsuka Long-Evans Tokushima Fatty rats (Kim *et al.*, 2003). In some Zucker studies heart weight is *decreased* relative to controls (Baynes *et al.*, 2009; Daniels *et al.*, 2012; Wang *et al.*, 2004), and the cardiac weight index can also be decreased in Zucker rats (van den Brom *et al.*, 2009). Defining a hypertrophic response in the Zucker model is particularly problematic due to maturational variations in body weight difference relative to lean controls. In other T2D models, cardiac weight *increased*, in GLUT-4 knockout mice (Domenighetti *et al.*, 2010; Huggins *et al.*, 2008; Kaczmarczyk *et al.*, 2003), the *db/db* mouse (Li *et al.*, 2006), and the *ob/ob* mouse (Dong *et al.*, 2007). This increase was maintained after normalisation in GLUT-4 knockout mice (Domenighetti *et al.*, 2010; Huggins *et al.*, 2008; Kaczmarczyk *et al.*, 2003) and the *db/db* mouse (Li *et al.*, 2006). Increased heart weight emerges only after normalisation in Goto-Kakizaki rats (Chandler *et al.*, 2007; Darmellah *et al.*, 2007), fructose-fed rats (Zhang *et al.*, 2011a) and in one study of Zucker diabetic fatty rats (Daniels *et al.*, 2012). In the T2D context, where cardiomyocyte size has been evaluated, unchanged (Daniels *et al.*, 2012; Howarth *et al.*, 2008; Mellor *et al.*, 2011) or increased dimensions are reported (Abel *et al.*, 1999; Domenighetti *et al.*, 2007; Dong *et al.*, 2007; Fredersdorf *et al.*, 2004; Li *et al.*, 2006; Zhang *et al.*, 2011b), or more rarely, decreased cell dimensions have been

observed (Howarth *et al.*, 2012). As with T1D studies, fewer studies in T2D were undertaken in females. In those that have investigated females without direct comparisons to males, heart weight was increased in GLUT-4 knockout mice (Abel *et al.*, 1999), *ob/ob* mice (Christoffersen *et al.*, 2003), *db/db* mice (Huynh *et al.*, 2012) and in a model of high fat, low STZ (Zhou *et al.*, 2011) or unchanged in a model of multiple low dose STZ (Howarth *et al.*, 2005). Increased normalised heart weight was also noted in *db/db* mice (Huynh *et al.*, 2012) and high fat, low STZ (Zhou *et al.*, 2011), while normalised heart weight was decreased in GLUT-4 knockout mice (Abel *et al.*, 1999).

Sex-specific comparisons in relation to hypertrophy occurrence have been made in a limited number of investigations, and are summarised in Table 1.2. In T1D, two studies of STZ treatment (rats, 5 week diabetes duration) report that both male and female animals achieve similar levels of hyperglycaemia, with no change in cardiac weight index in either sex (Rodrigues *et al.*, 1987; Tuncay *et al.*, 2007). In a mouse model of STZ (10 weeks diabetes), there was increased absolute heart weight in females but a reduction in cardiac weight indices in both sexes. This could be attributable to their increases observed in body weight (Bowden 2015). In the T2D (mild) sucrose-fed rat, a sex comparison of treatment did not find any differential responses, and there were no effects of diet on cardiac weight index in males or females (Cárdenas *et al.*, 2006). In contrast, an investigation of the genetic T2D Goto Kakazaki rat model identified a markedly greater increase in cardiac weight index in females, although hyperglycaemia was equivalent in both sexes (Desrois *et al.*, 2004). The females in this model exhibit more deranged plasma lipids than males, and it is possible that metabolic signals deriving from altered lipid handling have a greater role in mediating cardiac hypertrophy in female diabetics.

To summarise the diversity of findings from an extensive range of model types (involving either exclusively male cohorts or mixed sex groups), it is not possible to ascribe different observations regarding occurrence of cardiac hypertrophy to model-specific factors, or to the duration of treatment. Only a very small number of studies have pursued explicit sex comparisons, indicating that when hypertrophy occurs it is more pronounced in females (somewhat consistent with clinical findings). Much more work is required in this area and given that the Framingham study indicates that, after age, cardiac hypertrophy *per se* constitutes the most significant cardiac risk factor (Levy *et al.*, 1990), this should be an important area for future investigation.

1.3C Sex differences in diabetes-induced cardiac functional abnormalities

There have been extensive experimental efforts directed towards the characterisation of cardiac dysfunction in the progression of diabetic cardiomyopathy. Research has moved beyond the perception of diabetes as a single condition of glucose dysregulation, with attention now directed more towards distinguishing differential mechanisms in T1D and T2D which contribute to cardiomyopathy development (Isfort *et al.*, 2013). As discussed above, clinical investigations employing imaging techniques have established the occurrence of relatively early diastolic dysfunction, at a non-symptomatic stage, as a prelude to later progression to systolic dysfunction. In the experimental setting there is scope to dissect the components of cardiac diastolic and systolic dysfunction more precisely using different *in vivo* and *ex vivo* approaches. With only a few notable exceptions (highlighted at the end of each of the following sections, and presented in Table 1.2), these experiments have involved males only, mixed sex, or failed to specify sex these findings are included in Table 1.1).

Type 1 diabetes and cardiac function.

In males, impaired diastolic function *in vivo* is generally observed in rodent models of T1D (predominately STZ treated). Reduced echocardiographic E:A ratios, (Huynh *et al.*, 2010; Khong *et al.*, 2011; Meloni *et al.*, 2012; Ritchie *et al.*, 2012; Shao *et al.*, 2011; Ti *et al.*, 2011) and decreased end-diastolic volume (Couchevin *et al.*, 2008; Dong *et al.*, 2006) have been reported. Catheterisation of the left ventricle in males has revealed impaired/delayed relaxation (Hoit *et al.*, 1999; Huynh *et al.*, 2010; Strunz *et al.*, 2011; Ti *et al.*, 2011) and increased left ventricular end-diastolic pressures (Li *et al.*, 2011; Ritchie *et al.*, 2012), although such increases are not always identified (Huynh *et al.*, 2010). Analysis of pressure-volume relationships reveal increased left ventricular stiffness in males (Radovits *et al.*, 2009). *In vivo* assessment of animal T1D models has generally identified significant reduction of heart rate (Annapurna *et al.*, 2009; Hu *et al.*, 2011; Sharma *et al.*, 2011; Tian *et al.*, 2011; Xiao *et al.*, 2004), but not always (Hadour *et al.*, 1998; Shao *et al.*, 2011). This contrasts with observations of equivalent heart rate in diabetic and non-diabetic patients (Palmieri *et al.*, 2008), and may reflect greater glucose dysregulation in experimental models. Evidence of diastolic dysfunction (decreased E:A ratio) was found in young adult female STZ-treated rat. However, this study did not include male comparators (Joffe *et al.*, 1999; Yu *et al.*, 2007).

In vivo systolic dysfunction is also reported in many investigations of T1D in males, characterised by reduced ejection fraction as assessed via echocardiography (Sharma *et al.*, 2011; Ti *et al.*, 2011), reduced left ventricular systolic pressure development (Huynh *et al.*, 2010; Ritchie *et al.*, 2012; Xiao *et al.*, 2004), and a less steep end-systolic pressure-volume relationship (Radovits *et al.*, 2009), although bradycardia observed in this latter study will negatively influence contractility via the Treppe or staircase effect. In males, decreased maximum velocity of shortening has also been reported (Huynh *et al.*, 2010; Roe *et al.*, 2011), and fractional shortening may be decreased (Meloni *et al.*, 2012; Tian *et al.*, 2011) or unchanged (Hoit *et al.*, 1999; Huynh *et al.*, 2010; Strunz *et al.*, 2011; Ti *et al.*, 2011). Anaesthetic choice may be an important consideration in assessment of cardiac dysfunction by echocardiography. In the STZ model of diabetes, reduced cardiac output is observed with ketamine or etomidate, but not propofol (Crespo *et al.*, 2011).

In male *ex vivo* perfused hearts, evidence of systolic contractile dysfunction is usually evident with T1D. Reductions in systolic pressure and rate of pressure development have been reported in hearts from male STZ-treated animals (Nawata *et al.*, 2002; Ooie *et al.*, 2003; Ren *et al.*, 2011; Shimabukuro *et al.*, 1996; Takahashi *et al.*, 2003; Tosaki *et al.*, 1996) and in OVE26 mice, a less well characterised model of pancreatic β -cell dysfunction secondary to β -cell calmodulin overexpression (Xie *et al.*, 2011). In the *ex vivo* setting, while relaxation parameters can be very accurately assessed, it is difficult to establish the presence of diastolic dysfunction under baseline conditions. *Ex vivo* diastolic baseline pressure is standardised, although diastolic dysfunction can be assessed by examining end-diastolic pressure/volume relationships, and slope of relaxation. In studies of females, systolic function was decreased as assessed by isolated heart function in STZ-treated rats (Joffe *et al.*, 1999; Vadlamudi *et al.*, 1982) and reduced fractional shortening and cardiac output (Yu *et al.*, 2007).

Potential sex-specificity of cardiac function in T1D has received minimal attention, and available findings are summarised in Table 1.2. *Ex vivo* evaluation of young (6 weeks) STZ-treated rats identified less impairment of pressure development (+dP/dt) in female compared with male hearts (Rodrigues *et al.*, 1987). Other isolated heart studies in adult STZ-treated rats (12-14 weeks) show left ventricular pressure development is less adversely affected in females despite similar hyperglycaemia in both sexes (Tuncay *et al.*, 2007; Yaras *et al.*, 2007). *In vivo* functional analysis in CD1 mice revealed earlier

onset of diastolic dysfunction, with reduced E/A ratio, in female diabetic mice at 8 weeks post STZ induction, despite similar levels of increase in blood glucose in both sexes (Moore *et al.*, 2014).

At present, an experimental correlate of the increased female jeopardy observed clinically in T1D is observed to some extent, in particular, available experimental evidence does not address the critical issue of diastolic dysfunction, with exception of one study in the literature as identified in previous sections. It may be that relatively short-term STZ treatment (without insulin supplement) in female rodents does not recapitulate the clinical state, and that extended duration studies are required.

Type 2 diabetes and cardiac function

In males, diastolic dysfunction, evaluated via *in vivo* echocardiography (altered E:A ratio), is observed in Zucker rats (van den Brom *et al.*, 2009), Otsuka Long-Evans Tokushima Fatty rats (Semeniuk *et al.*, 2002) and leptin deficient (*ob/ob*) mice (Christoffersen *et al.*, 2003). Diastolic dysfunction could not be identified echocardiographically in *db/db* males. The E:A ratio can appear normal while hearts transition from mild (elevated E:A ratio) to severe (decreased E:A ratio) diastolic dysfunction (Galderisi, 2006), and a single time-point determination may not be definitive. Millar catheter assessment of left ventricular function is suggestive of diastolic and systolic dysfunction in male Zucker rats (van den Brom *et al.*, 2009), though reduced heart rate in these rats may contribute to altered contractility. Millar catheter assessment of male *db/db* mice did not identify diastolic dysfunction (Panagia *et al.*, 2007). A similar lack of effect on diastolic function has been observed in male GLUT-4 knockout mice (Abel *et al.*, 1999), Zucker diabetic fatty rats (Wang *et al.*, 2005), and in sucrose fed rats (Vasanji *et al.*, 2006), though evidence of increased left ventricular stiffness in male Zucker rats has also been revealed by examining changes in the relationship between pressure and volume (Radovits *et al.*, 2009). Diastolic dysfunction was evident as a reduced slope of relaxation in hearts isolated from GLUT-4 knockout mice (Huggins *et al.*, 2008), *ob/ob* mice (Li *et al.*, 2006), *db/db* mice (Dong *et al.*, 2007) and fructose-fed rats (Chang *et al.*, 2007).

In studies investigating female mice, diabetes-induced diastolic dysfunction was evident in female (*db/db*) mice (Christoffersen *et al.*, 2003; Huynh *et al.*, 2012), female *ob/ob* mice (Zibadi *et al.*, 2011) and in female mice fed a high-fat diet combined with low

dose STZ (Zhou *et al.*, 2011), but not in the female GLUT-4 knockout mouse (Abel *et al.*, 1999).

In males, systolic dysfunction *in vivo* has been identified in numerous T2D models. High-fat diet treatment in male rats was observed to induce reduced fractional shortening and ejection fraction (Ouwens *et al.*, 2007). In male *db/db* mice, left ventricular developed pressure was decreased in one study (Khalid *et al.*, 2011), and fractional shortening found to be decreased in others (Carley *et al.*, 2004; Park *et al.*, 2009; Semeniuk *et al.*, 2002; Vecoli *et al.*, 2011). The male Torii (spontaneous non-obese diabetic, Sprague-Dawley derived) rat exhibits reduced ejection fraction and fractional shortening (Lakshmanan *et al.*, 2012), and the male Goto-Kakizaki rat is reported to display reduced fractional shortening at 8 but not 20 weeks (Chandler *et al.*, 2007). In male Otsuka Long-Evans Tokushima Fatty rats, fractional shortening was not reduced (even though diastolic dysfunction was observed) (Kim *et al.*, 2003). The male Zucker rat exhibited decreased cardiac output, however coincident reductions in heart rate complicate interpretation of changes in contractile measures (Daniels *et al.*, 2012). Male mice fed a high-fat diet from birth exhibit decreased ejection fraction and elevated end-systolic volumes suggestive of systolic dysfunction (Louwe *et al.*, 2012b). No difference in systolic function was detected echocardiographically in male *ob/ob* (Mascareno *et al.*, 2009) *db/db* (Panagia *et al.*, 2007) and Goto-Kakizaki diabetic rat (Chandler *et al.*, 2007).

Heart rate reduction in T2D development has not been generally identified. Some studies in male Zucker rats report no changes in heart rate (Baynes *et al.*, 2009; Chen *et al.*, 2010; VanHoose *et al.*, 2010), while others report significant heart rate reductions (Daniels *et al.*, 2012; Fredersdorf *et al.*, 2004; van den Brom *et al.*, 2009; Wang *et al.*, 2004). Heart rate was also not reduced in male leptin-receptor deficient (*db/db*) mice (Khalid *et al.*, 2011), leptin deficient (*ob/ob*) mice, (Ge *et al.*, 2012; Mascareno *et al.*, 2009), and male mice fed a high-fat diet (Louwe *et al.*, 2012b). Male Goto-Kakizaki rat heart function was examined in un-paced, isolated hearts and both reduced and unchanged rate measures were obtained (Desrois *et al.*, 2010; Desrois *et al.*, 2004). The mechanisms underlying this disparity in outcomes are unclear.

Ex vivo experiments assessing isolated heart function of T2D models also reveal reduced left ventricular pressure development, contractility and lusitropy in isolated hearts from leptin-receptor deficient male (*db/db*) mice (Belke *et al.*, 2004; Vecoli *et al.*,

2011). Isolated working *db/db* hearts also display reduced contractility (cardiac output, cardiac work and left ventricular pressure development), although reduced heart rate in male diabetic mice likely contributes to contractility differences in such experiments (Hafstad *et al.*, 2007; Hafstad *et al.*, 2006). Isolated hearts from male Zucker rats (Hafstad *et al.*, 2007; Wang *et al.*, 2004) were found to perform less contractile work, likely due in part to reduced heart rate. Hearts isolated from male Goto-Kakizaki rats demonstrated reduced systolic function despite comparable heart rates (Desrois *et al.*, 2010) while GLUT-4 knockout mice do not demonstrate reduced contractile function (Abel *et al.*, 1999). In females, reduced fractional shortening and ejection fraction was observed in a high-fat/low dose STZ model of T2D (Zhou *et al.*, 2011), but was unchanged in female *db/db* mice as assessed by echocardiography (Christoffersen *et al.*, 2003; Huynh *et al.*, 2012) and was also unchanged in female GLUT-4 knockout mice (Abel *et al.*, 1999).

Sex-specific comparisons in T2D models, as for T1D models, are also modest in number (Table 1.1). A high-fat diet (45%) for 12 weeks in rats induced equivalent weight gain in both sexes, but hyperinsulinemia, hyperglycaemia and functional decrement (reduction in ejection fraction) were only observed in males (Louwe *et al.*, 2012b). In a different rat dietary study, fructose feeding for 8 months produced a more marked reduction in *ex vivo* cardiac contractile function in females (measured as heart rate x pressure product), although glycaemic status and heart rate reduction was the same for both sexes (Cárdenas *et al.*, 2005; Cárdenas *et al.*, 2006). This differential was not evident earlier in disease development, and age may be an important factor in recapitulating diabetic functional effects in experimental settings. As noted above, diastolic dysfunction was observed in female leptin-receptor deficient (*db/db*) mice (Huynh *et al.*, 2012) but was not established in a different study involving *db/db* males (Park *et al.*, 2009). This possible sex difference requires further verification.

It is apparent that from an experimental perspective, availability of information about the comparative functional attributes of T2D male and female hearts is limited. The diversity of experimental models is itself a challenge, and it may be that for T2D, cardiopathology development takes on sexually dimorphic characteristics only in more mature animals. Current knowledge does not permit conclusive statements. Much more information is required to begin to make informed links between clinical and experimental T2D disease differences in males and females.

1.3D Ischemia reperfusion - structural and functional injury in experimental setting

Ischemic heart disease is the most common form of cardiovascular disease in western society (Vaccarino *et al.*, 2011). Despite established clinical evidence demonstrating increased occurrence of acute myocardial infarction in diabetes and increased sensitivity of the diabetic heart to ischemia, experimental models have failed to recapitulate this scenario consistently. Experimentally, ischemic resilience of the heart has been investigated at multiple levels: *in vivo*, in the isolated *ex vivo* heart, and using isolated single cardiomyocytes.

Most models of STZ treatment in rats have shown *less susceptibility* to ischemia-reperfusion injury under *in vivo* (Ma *et al.*, 2006; Ravingerova *et al.*, 2003) and *ex vivo* conditions (Nawata *et al.*, 2002; Ooie *et al.*, 2003; Shi-ting *et al.*, 2010). Three studies attributed this “protective status” to acute STZ induction (45-60 mg/kg STZ; 1-4 weeks diabetes), showing loss of “protection” and *no difference* in ischemic vulnerability when the diabetic duration was increased to a longer, chronic period (6-8 weeks) (Ma *et al.*, 2006; Ravingerova *et al.*, 2003; Shi-ting *et al.*, 2010). However, not all chronic studies are in agreement with this. Chronic administration of STZ (65 mg/kg STZ; 12 weeks diabetes) in rats has shown increased left ventricular function, reduced ventricular fibrillation and creatinine kinase release. *In vitro* incubation of rat neonatal cardiomyocytes incubated in high glucose media (25 mM) for 3 days and subjected to 1 hour ischemia in a hypoxic chamber (2.3% O₂-5% CO₂) was associated with reduction in necrosis, apoptosis and calcium content and lesser damage (Schaffer *et al.*, 2000). Few T1D studies have shown *increased susceptibility* to ischemic damage when STZ was administered in combination of other glucotoxic analogues or increased in dosage. T1D-induced in dogs (STZ 25 mg/kg and Alloxan 40 mg/kg) (Kersten *et al.*, 2000) and STZ rats (70 mg/kg) (Marfella *et al.*, 2002) have shown increased infarct size, even in acute STZ settings (9 days-3 weeks). Therefore, there is some indication of improved structural functional recovery in T1D rodent models, though this phenomenon is largely dependent on duration and dosage of STZ treatment. Sex aspects of post-ischemic recovery in T1D are yet to be explored.

Unlike T1D, limited ischemia-reperfusion studies using T2D models have been conducted in *in vivo* experiments. Langendorff studies from Zucker diabetic fatty and Goto-Kakizaki (GK) rats have reported *smaller infarct size* (*vs.* controls) with

respective 50 mins (Kristiansen *et al.*, 2004) or 30 mins (Tsang *et al.*, 2005) regional ischemia followed by 2hrs reperfusion. However, this lesser susceptibility to ischemic injury in GK rats has been observed consistently. *No differences* in ventricular contraction rate were observed in aged GK male rats perfused with Krebs-Hensleit buffer under a Langendorff set up (Desrois *et al.*, 2004). A follow up study from the same group using similar experimental settings reported *increased susceptibility to ischemic damage* when additional fatty acid substrates were included in the perfusate to mimic the *in vivo* scenario better (Desrois *et al.*, 2010). This suggested that *in vivo* components such as other energy substrates, platelets and neutrophils could be key in mediating cell damage in T2D settings. An *in vivo* study on *db/db* mice with non-recovery 30 mins regional ischemia and 2 hours reperfusion showed increased infarct size (Jones *et al.*, 1999). Interestingly, sex differences gleaned from the earlier study by Desrois and colleagues depicted female hearts with larger infarct areas when there were no differences in males (Desrois *et al.*, 2004). This suggests that a smaller increase in extracellular glucose was sufficient to cause a signalling response in female T2D. Therefore, it is difficult to gauge ischemic damage sensitivity in T2D heart from studies, which have been predominantly conducted in *ex vivo* settings. The usage of a variety of genetic models has also added a further degree of complication in interpretation of results.

Therefore, clinical observations of increased acute myocardial infarction in diabetics are not always observed in experimental settings. Experimental T1D models have mostly shown enhanced recovery after ischemia and insights from T2D models have been inconclusive. Studies examining sex differences in post-ischemic functional recovery using animal models are very limited. A review by Paulson *et al.* (2007) attributed these controversial findings in the experimental setting to differences in duration of diabetes, usage of glucose as the only substrate and a no-flow ischemia protocol (Paulson, 1997). Lack of other comorbidities such as age, dyslipidaemia and hypertension could be causative in this discrepancy (Whittington *et al.*, 2012). Interestingly, enhanced recovery in the diabetic setting has been associated with altered cardiac glycogen substrate utilisation in ischemic stress, reduced glycolytic rates and reduced accumulation of lactate resulting in enhanced recovery post-ischemia conditions (Nawata *et al.*, 2002; Ooie *et al.*, 2003; Shi-ting *et al.*, 2010). Indeed, we have demonstrated previously that a non-pathological metabolic stress of 48 hours fasting

leads to selective glycogen accumulation in female hearts and altered glycogen-phagic processing (Reichelt *et al.*, 2013b). This alteration in glycogen/glucose regulation in the diabetic heart will be the focus in the next section.

Table 1.1: Cardiac morphology and functional characteristics in experimental models of T1D and T2D (male only, female only or pooled sex studies)

Parameter	Sex	Effect	References	
			T1D	T2D
Heart weight	F	↑	(Ceylan-Isik <i>et al.</i> , 2006b)	(Abel <i>et al.</i> , 1999), (Christoffersen <i>et al.</i> , 2003), (Huynh <i>et al.</i> , 2012), (Zhou <i>et al.</i> , 2011)
		↓	(Vadlamudi <i>et al.</i> , 1982), (Yu <i>et al.</i> , 2007)	
		↔	(Howarth <i>et al.</i> , 2005)	(Howarth <i>et al.</i> , 2005)
	M	↑	(Ceylan-Isik <i>et al.</i> , 2006b)	(Domenighetti <i>et al.</i> , 2010), (Huggins <i>et al.</i> , 2008), (Li <i>et al.</i> , 2006), (Dong <i>et al.</i> , 2007), (Abel <i>et al.</i> , 1999)
		↓	(Ooie <i>et al.</i> , 2003), (Hu <i>et al.</i> , 2011), (Ren <i>et al.</i> , 2000), (Le Douairon Lahaye <i>et al.</i> , 2012), (Singh <i>et al.</i> , 2006), (Raimondi <i>et al.</i> , 2004), (Nawata <i>et al.</i> , 2002) ^a , (Takahashi <i>et al.</i> , 2003) ^a , (Xie <i>et al.</i> , 2011), (Tosaki <i>et al.</i> , 1996)	(Baynes <i>et al.</i> , 2009), (Wang <i>et al.</i> , 2004), (Daniels <i>et al.</i> , 2012), (Chandler <i>et al.</i> , 2007) [†]
		↔	(Wold <i>et al.</i> , 2006), (Wold <i>et al.</i> , 2001), (Roe <i>et al.</i> , 2011), (Ritchie <i>et al.</i> , 2012), (Shimabukuro <i>et al.</i> , 1996), (Hoit <i>et al.</i> , 1999) ^a , (Huynh <i>et al.</i> , 2010),	(Howarth <i>et al.</i> , 2008), (Panagia <i>et al.</i> , 2007), (Vasanji <i>et al.</i> , 2006), (Fredersdorf <i>et al.</i> , 2004), (Wang <i>et al.</i> , 2005), (Howarth <i>et al.</i> , 2012), (Mellor <i>et al.</i> , 2011), (Mascareno <i>et al.</i> , 2009) [†] , (Park <i>et al.</i> , 2009), (Ouwens <i>et al.</i> , 2007) [†] , (Kim <i>et al.</i> , 2003), (Hafstad <i>et al.</i> , 2006), (Chess <i>et al.</i> , 2007), (Chang <i>et al.</i> , 2007) [†]
	NS	↓	(Silva <i>et al.</i> , 2011) ^a	
	F	↑	(Vadlamudi <i>et al.</i> , 1982), (Ceylan-Isik <i>et al.</i> , 2006b), (Joffe <i>et al.</i> , 1999) [†]	(Huynh <i>et al.</i> , 2012), (Zhou <i>et al.</i> , 2011)
		↓		(Abel <i>et al.</i> , 1999),
		↔	(Yu <i>et al.</i> , 2007)	
Cardiac weight index	M	↑	(Ooie <i>et al.</i> , 2003), (Ren <i>et al.</i> , 2011), (Hu <i>et al.</i> , 2011), (Ren <i>et al.</i> , 2000), (Wold <i>et al.</i> , 2001), (Bollano <i>et al.</i> , 2007), (Le Douairon Lahaye <i>et al.</i> , 2012), (Ceylan-Isik <i>et al.</i> , 2006b), (Ti <i>et al.</i> , 2011), (Nawata <i>et al.</i> , 2002) ^a , (Hoit <i>et al.</i> , 1999) ^a	(Zhang <i>et al.</i> , 2011a), (Kaczmarczyk <i>et al.</i> , 2003), (Domenighetti <i>et al.</i> , 2010), (Huggins <i>et al.</i> , 2008), (Li <i>et al.</i> , 2006), (Darmellah <i>et al.</i> , 2007) ^a , (Daniels <i>et al.</i> , 2012), (Chandler <i>et al.</i> , 2007) [†]
		↓	(Khong <i>et al.</i> , 2011), (Tosaki <i>et al.</i> , 1996)	(Panagia <i>et al.</i> , 2007), (Abel <i>et al.</i> , 1999), (van den Brom <i>et al.</i> , 2009) [†]
		↔	(Wold <i>et al.</i> , 2006), (Raimondi <i>et al.</i> , 2004), (Roe <i>et al.</i> , 2011), (Ritchie <i>et al.</i> , 2012), (Takahashi <i>et al.</i> , 2003) ^a , (Xie <i>et al.</i> , 2011), (Huynh <i>et al.</i> , 2010)	(Howarth <i>et al.</i> , 2012), (Mellor <i>et al.</i> , 2011), (Baynes <i>et al.</i> , 2009), (Wang <i>et al.</i> , 2004), (Kim <i>et al.</i> , 2003), (Chang <i>et al.</i> , 2007) [†] , (Chess <i>et al.</i> , 2007)
	M/F	↑		(Ritchie <i>et al.</i> , 2007)
		↔		(Daniels <i>et al.</i> , 2011)
	NS	↑	(Silva <i>et al.</i> , 2011) ^a	
	F	↑		(Abel <i>et al.</i> , 1999), (Huynh <i>et al.</i> , 2012)
		↔	(Ceylan-Isik <i>et al.</i> , 2006b), (Hofmann <i>et al.</i> , 1995), (Howarth <i>et al.</i> , 2005)	
		↑	(Ti <i>et al.</i> , 2011), (Wold <i>et al.</i> , 2006), (Li <i>et al.</i> , 2011), (Khong <i>et al.</i> , 2011), (Ritchie <i>et al.</i> , 2012), (Huynh <i>et al.</i> , 2010)	(Fredersdorf <i>et al.</i> , 2004), (Domenighetti <i>et al.</i> , 2010), (Dong <i>et al.</i> , 2007), (Abel <i>et al.</i> , 1999), (Zhang <i>et al.</i> , 2011a), (Li <i>et al.</i> , 2006)
Cardio-myocyte size	M	↓		(Howarth <i>et al.</i> , 2012)
		↔	(Ceylan-Isik <i>et al.</i> , 2006b), (Hofmann <i>et al.</i> , 1995), (Kotsanas <i>et al.</i> , 2000), (Raimondi <i>et al.</i> , 2004), (Shao <i>et al.</i> , 2011), (Roe <i>et al.</i> , 2011), (Shao <i>et al.</i> ,	(Howarth <i>et al.</i> , 2008), (Mellor <i>et al.</i> , 2011), (Daniels <i>et al.</i> , 2012)

		2010)	
	NS	↔	(Daniels <i>et al.</i> , 2011)
		↓	(Silva <i>et al.</i> , 2011)
	F	↓	(Joffe <i>et al.</i> , 1999) [#] (Yu <i>et al.</i> , 2007) [#]
		↔	(Christoffersen <i>et al.</i> , 2003) [#] , (Huynh <i>et al.</i> , 2012) [#] , (Zhou <i>et al.</i> , 2011) [‡]
			(Abel <i>et al.</i> , 1999) [‡] , (Zibadi <i>et al.</i> , 2011) [#]
		↓	(Huynh <i>et al.</i> , 2010) [#] , (LaRocca <i>et al.</i> , 2012) [#] , (Ti <i>et al.</i> , 2011) [#] , (Shao <i>et al.</i> , 2011) [#] , (Meloni <i>et al.</i> , 2012) [#] , (Xiao <i>et al.</i> , 2004) [#] , (Sharma <i>et al.</i> , 2011) [#] , (Khong <i>et al.</i> , 2011) [#] , (Radovits <i>et al.</i> , 2009) ^{#**} , (Ritchie <i>et al.</i> , 2012) [#] , (Xie <i>et al.</i> , 2011) [#] , (Strunz <i>et al.</i> , 2011) [#] , (Ren <i>et al.</i> , 2011) [‡] , (Li <i>et al.</i> , 2011) [#]
	M		(Wang <i>et al.</i> , 2004) [‡] , (Daniels <i>et al.</i> , 2012) [#] , (Huggins <i>et al.</i> , 2008) [‡] (at 60w only), (Li <i>et al.</i> , 2006) [‡] , (Dong <i>et al.</i> , 2007) [‡] , (van den Brom <i>et al.</i> , 2009) [#] , (Semeniuk <i>et al.</i> , 2002) [#] , (Lakshmanan <i>et al.</i> , 2012) [‡] , (Khalid <i>et al.</i> , 2011) [‡] , (Belke <i>et al.</i> , 2004) [‡] , (Chang <i>et al.</i> , 2007) [#] , (Darmellah <i>et al.</i> , 2007) [#] , (Baynes <i>et al.</i> , 2009) [#]
		↔	(Shimabukuro <i>et al.</i> , 1996) [‡]
			(Radovits <i>et al.</i> , 2009) [#] , (Panagia <i>et al.</i> , 2007) [‡] , (Wang <i>et al.</i> , 2005) [‡] , (Vasanji <i>et al.</i> , 2006) [#] , (Chandler <i>et al.</i> , 2007) [#] , (Park <i>et al.</i> , 2009) [#] , (Abel <i>et al.</i> , 1999) [‡] , (Ouwens <i>et al.</i> , 2007) [#] , (Kim <i>et al.</i> , 2003) [#]
	M/F	↔	(Hafstad <i>et al.</i> , 2007) [‡]
	NS	↓	(Vecoli <i>et al.</i> , 2011) [‡]
	F	↓	(Vadlamudi <i>et al.</i> , 1982) [‡] , (Yu <i>et al.</i> , 2007) [‡] , (Joffe <i>et al.</i> , 1999) [#]
		↔	(Zhou <i>et al.</i> , 2011) [#]
		↑	(Christoffersen <i>et al.</i> , 2003) [#] , (Huynh <i>et al.</i> , 2012) [#] , (Abel <i>et al.</i> , 1999) [‡]
			(Fredersdorf <i>et al.</i> , 2004)
		↓	(Ooie <i>et al.</i> , 2003) [#] , (LaRocca <i>et al.</i> , 2012) [#] , (Shao <i>et al.</i> , 2010) [#] , (Ti <i>et al.</i> , 2011) [#] , (Li <i>et al.</i> , 2011) [#] , (Meloni <i>et al.</i> , 2012) [#] , (Xiao <i>et al.</i> , 2004) [#] , (Roe <i>et al.</i> , 2011) [#] , (Sharma <i>et al.</i> , 2011) [#] , (Radovits <i>et al.</i> , 2009) [#] , (Nawata <i>et al.</i> , 2002) [#] , (Takahashi <i>et al.</i> , 2003) [#] , (Xie <i>et al.</i> , 2011) [#] , (Strunz <i>et al.</i> , 2011) [#] , (Tosaki <i>et al.</i> , 1996) [‡] , (Ren <i>et al.</i> , 2011) [‡]
	M		(Wang <i>et al.</i> , 2004) [‡] , (Wang <i>et al.</i> , 2005) [‡] , (Vasanji <i>et al.</i> , 2006) [#] , (Daniels <i>et al.</i> , 2012) ^{***} , (Huggins <i>et al.</i> , 2008) [‡] , (Li <i>et al.</i> , 2006) [‡] , (Dong <i>et al.</i> , 2007) [‡] , (van den Brom <i>et al.</i> , 2009) [#] , (Park <i>et al.</i> , 2009) [#] , (Ouwens <i>et al.</i> , 2007) [#] , (Semeniuk <i>et al.</i> , 2002) [#] , (Carley <i>et al.</i> , 2004) [#] , (Lakshmanan <i>et al.</i> , 2012) [#] , (Khalid <i>et al.</i> , 2011) [#] , (Belke <i>et al.</i> , 2004) [‡] , (Hafstad <i>et al.</i> , 2006) ^{4**} , (Darmellah <i>et al.</i> , 2007) [#]
		↔	(Bollano <i>et al.</i> , 2007) [#] , (Huynh <i>et al.</i> , 2010) [#] , (Khong <i>et al.</i> , 2011) [#] , (Ritchie <i>et al.</i> , 2012) [#] , (Shimabukuro <i>et al.</i> , 1996) [#] , (Hoit <i>et al.</i> , 1999) [#]
			(Radovits <i>et al.</i> , 2009) [#] , (Panagia <i>et al.</i> , 2007) [#] , (Mascareno <i>et al.</i> , 2009) [#] , (Chandler <i>et al.</i> , 2007) [#] , (Abel <i>et al.</i> , 1999) [‡] , (Kim <i>et al.</i> , 2003) [#] , (Baynes <i>et al.</i> , 2009) [#]
	M/F	↓	(Hafstad <i>et al.</i> , 2007) ^{***}
		↔	(Daniels <i>et al.</i> , 2011) [#]
	NS	↓	(Tian <i>et al.</i> , 2011) [#]
			(Vecoli <i>et al.</i> , 2011) [#]

Abbreviations: F, data from females; M, data from males; M/F, data pooled from male and female data; NS, sex not specified;

Arrows specify alterations relative to age-matched, control animals

↑, diabetes-induced increase; ↓, diabetes-induced decrease; ↔, no effect of diabetes;

* heart weight derived from calculations and not statistically assessed;

** bradycardia will impact on cardiac function;

† left ventricular values alone; cardiac weight index based on LV/tibia or LV/BWT.

^a Based on bi-ventricular weight

[#] *in vivo* function assessed

[‡] *ex vivo* function assessed

Table 1.2: Sex-specific features of the cardiac response to T1D and T2D in experimental models (direct sex comparisons only)

Type	Animal Model	Model	Duration (weeks)	Age at expt (weeks)	Blood Glucose			HW			HW:BW			Ventricular developed pressure			Ventricular rate of contraction				Reference
					Female	Male	F vs M	Female	Male	F vs M	Female	Male	F vs M	Female	Male	F vs M	Female	Male	F vs M		
T1D	Rat	STZ	6	18-20	↑	↑	=				↔	↔	=	↓	↓	-	↓	↓	-	†	(Tuncay <i>et al.</i> , 2007)
	Rat	STZ	6	15	↑	↑	+	=	↓	-	=	↑	-				↓	↓	-	†	(Rodrigues <i>et al.</i> , 1987)
	Rat	STZ	5	?	↑	↑	=							↓	↓	-	↓	↓	-		(Yaras <i>et al.</i> , 2007)
T2D	Rat	GK	genetic	52	↑	↑	=	↑	↔	+	↑	↑	+	↔	↔	=				*	(Desrois <i>et al.</i> , 2004)
	Rat	Sucrose	13	17	↔	↔	=	↔	↔	=							↔	↔	=	*	(Cárdenas <i>et al.</i> , 2006)
			22	26	↔	↔	=	↔	↔	=						↔	↓	-	*		
			35	39	↔	↔	=	↔	↔	=						↓	↓	=	*		
	Rat	Sucrose	35	40													↔	↓	-	‡	(Cárdenas <i>et al.</i> , 2005)
	Mouse	High-fat	5-6	18-22	↔	↑	-										↔	↔	=		(Louwe <i>et al.</i> , 2012a)
			11-12	24-28	↔	↑	-										↔	↔	=		

Table entries consist of studies involving male and female experimental groups (rodent strains; Rat, Wistar; Mouse, C57Bl6).

Abbreviations: Expt, experiment; vs, versus; T1D, type 1 diabetes; T2D, type 2 diabetes; HW, heart weight; HW:BW, Heart weight normalised to bodyweight; STZ, streptozotocin; M diabetes-induced change in male; F, diabetes-induced change in female; F vs M, extent of change in female compared to male; GK Goto-Kakizaki;

Symbols indicate alterations relative to age-matched, control animals.

↑, diabetes-induced increase; ↓, diabetes-induced decrease; ↔, no effect of diabetes;

+, effect was greater in females; -, effect was lesser in females.

† Inferred from data presented (not directly compared in study);

* Contractility will be influenced by alterations in heart rate;

‡ Heart rate not reported

1.4 RAS/AngII involvement in diabetic cardiomyopathy

Upregulation of cardiac renin-angiotensin system (RAS) has been implicated in as a key contributor to underlying diastolic dysfunction and fibrotic remodelling in diabetes (Lim *et al.*, 2004; Singh *et al.*, 2008; Velloso *et al.*, 2006). The RAS is essential for increasing sodium and fluid absorption when the body is challenged by sodium restriction or fluid loss. Renin secretion is triggered in the kidney following reduced sodium delivery to the distal nephron. Renin converts angiotensinogen from the liver to Angiotensin I (AngI), which is largely inactive and is converted to active Angiotensin II (AngII) by AngI Converting Enzyme (ACE). AngII is known to interact with at least two distinct receptor subtypes, designated AT1 and AT2, both of which belong to the superfamily of seven transmembrane receptors. The two receptors share little homology (~34% amino acid sequence identity) and exhibit little resemblance in relation to signalling mechanisms (de Gasparo *et al.*, 2000). AngII activates the AngII Type 1 (AT1) receptor to produce intense vasoconstriction and raises blood pressure while activation of the AngII Type 2 (AT2) receptor produces vasodilation. AngII also stimulates aldosterone secretion from glomerulosa cells via AT1 to increase sodium chloride and water reabsorption through mineralocorticoid receptor (MR) activation (Luther *et al.*, 2011). Moreover, AngII also plays a role in regulating long-term responses, including growth and remodelling effects in cardiac, renal and vascular tissue (de Gasparo *et al.*, 2000).

Ganten and colleagues demonstrated, for the first time, that all components of RAS can be produced locally in several organs and tissues, four decades ago (Ganten *et al.*, 1971). Since then, local RAS has been found in the heart (Zhang *et al.*, 1995), blood vessels (Lavoie *et al.*, 2003), pancreas (Sernia, 2001), central nervous system (Nielsen *et al.*, 2000), reproductive system (Vinson *et al.*, 1997) and adipose tissue (Engeli *et al.*, 2000). The local systems appear to be regulated independently of the circulatory RAS but can also interact with the latter functioning as an intracrine, paracrine and endocrine mediator of cellular growth, extracellular matrix formation, vascular proliferation, endothelium function and apoptosis (Miyazaki *et al.*, 2006; Paul *et al.*, 2006). All components of the RAS have shown to be locally expressed in the heart (Zhang *et al.*, 1995). Renin and angiotensinogen mRNA levels are low in normal hearts (Dzau, 1993), but ACE mRNA is readily detectable (Yamada *et al.*, 1998). Cardiac AngI and AngII

levels, on the other hand, can achieve levels two to three times higher than the levels in plasma (Danser *et al.*, 1994). Cardiac AngII levels increase in response to pressure and volume overload (De Mello *et al.*, 2000). Pathophysiological elevation of local cardiac AngII has been shown to induce hypertrophy and contractile abnormalities in humans and rodents (Domenighetti *et al.*, 2005; Mazzolai *et al.*, 2000; Singh *et al.*, 2008). The role of cardiac AngII in the pathogenesis of diabetic cardiomyopathy in a setting of normotensive cardiac hypertrophy is yet to be elucidated.

1.4A Upregulation of RAS in the diabetic heart

The link between RAS and diabetes became apparent with studies in T1D and T2D demonstrating that RAS inhibition significantly reduced the risk of developing insulin resistance (Yamada *et al.*, 1998; Yusuf *et al.*, 2001) and lowered the incidence of vascular complications in diabetic patients (Brenner *et al.*, 2001; Jandeleit-Dahm *et al.*, 2005; Lewis *et al.*, 2001; Parving *et al.*, 2001) and in animal models (Chu *et al.*, 2006; Nakayama *et al.*, 2005). Upregulation of the activity of RAS and diabetes are frequently observed as coincidental occurrences in the plasma of diabetic patients (Lim *et al.*, 2004; Velloso *et al.*, 2006) and in fructose-fed T2D rats (Kamide *et al.*, 2002). Other experimental studies on fructose-fed mice have shown increased oxidative damage and endothelial cell apoptosis, autophagy and necrosis followed by interstitial fibrosis (Mellor *et al.*, 2011).

AngII is well known to be pro-growth and pro-fibrotic. It acts on the AT1 receptor to activate MAP Kinase signalling to induce pathological growth. AngII stimulation of TGF β for fibroblast collagen production and collagen accumulation is evident in *db/db* T2D models (Huynh *et al.*, 2012; Singh *et al.*, 2008) and STZ-induced T1D models (Connelly *et al.*, 2007b; Shiomi *et al.*, 2003). Beyond the role in collagen deposition consequent to myocyte loss, recent advances in autophagy studies have identified AngII as a myocardial autophagic modulator. Neonatal cardiomyocytes expressing AngII AT1 receptor exhibited increased autophagy and co-expression of AT1 and AT2 receptors completely abrogated this AngII-AT1-mediated autophagic response. This suggests the involvement of these G-protein coupled receptors in autophagy induction (Porrello *et al.*, 2009). While upregulation of RAS is often reported in diabetic patients and animal models, the specific role of local cardiac RAS in diabetes is not fully understood.

1.4B Possible signalling crosstalk between AngII and insulin signalling pathway

Possible cross-talk between these pathways has been suggested as AngII adversely affects glucose metabolism and decreases its uptake and utilisation by interfering with insulin-mediated glucose uptake (Cooper *et al.*, 2007; Olivares-Reyes *et al.*, 2009). AngII activates NADPH oxidase and increases reactive oxygen species (ROS) generation through the AT1 receptor. Subsequent activation of redox-sensitive serine kinases such as c-jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) leads to decreased activation of protein kinase B (Akt) and translocation of glucose transporter GLUT4 to the membrane. Blockade of RAS at various steps, ie. AT1 receptor or mineralocorticoid (MR) and ACE, ameliorates these effects (Luther *et al.*, 2011). Further investigation is warranted to understand the mechanistic interaction between insulin signalling and AngII pathway.

Some sex-specific insights on RAS and diabetes are available from both clinical and experimental setting. A meta-analysis of clinical trials have shown a consistent trend towards greater benefit of ACE inhibitors in men than women (Rabi *et al.*, 2008). Women with a higher a risk of heart failure had a coincidental lower prescription of RAS inhibitors and were more frequently prescribed digitalis and diuretics (Agvall *et al.*, 2001; Chin *et al.*, 1997; Mejhert *et al.*, 1999). Some experimental evidence of sex differences in RAS involvement is available from diabetic rats. Experimentally, sex differences have also been reported in the expression of various components of RAS (“classical” and “non-classical”) in non-diabetic cardiac pathology (Dalpiaz *et al.*, 2015). Male rats had a higher AngII content compared to their non-diabetic controls, while diabetic female rats maintained similar levels as their controls. Interestingly, ovariectomised female diabetics had similar levels of AngII content as male diabetics (Shimoni *et al.*, 2004). Estrogen has also been shown to modulate various components of RAS. An estrogen response element located at the promoter region of *AOGN* gene may be instrumental in regulating local tissue AngII production (Feldmer *et al.*, 1991). Therefore, these studies indicate that females were less susceptible to the diabetes-induced elevation of cardiac AngII. Whether this plays a role in the sex differences observed in selective T1D and T2D cardiac pathology is still unknown.

1.5 Cardiac glycogen abnormalities in the diabetic heart

Glycogen is a large complex polysaccharide of glucose residues linked in chains via α -1,4-glycosidic bonds, with branching occurring via α -1,6-glycosidic bonds (Figure 1.1) (Larner *et al.*, 1952). Glycogen exists as either small β particles (less than ~50 nm) or as larger α particles up to 300 nm in diameter that consist of many β particles aggregating together by an unknown mechanism (Drochmans, 1962; Ryu *et al.*, 2009). Electron micrographs of purified cardiac glycogen reveal that glycogen is stored as both α and β particles in the heart, which may reflect the necessity for a steady glucose supply with additional capacity for rapid increases in workload (Besford *et al.*, 2012; Reichelt *et al.*, 2013b). The structure-function relationships pertaining to glycogen storage and usage have yet to be determined. Interestingly, in diabetes, up to a four-fold increase in glycogen has been observed in adult hearts, contributing to specific cardiac glycogen pathology (Alfarano *et al.*, 2011).

1.5A Altered myocardial metabolism and glycogen accumulation in diabetes

Metabolic stress as a result of substrate imbalance is a key feature of diabetic cardiomyopathy. Under normal conditions, the myocardium primarily relies on fatty acids (50-70%) and glucose (30-50%) as metabolic substrates. In diabetes, an apparent shift towards a higher reliance on fatty acids (90-99%) is evident for ATP production. This is primarily due to lower glucose oxidation resulting from reduced insulin-dependent glucose uptake and as result of increased circulating fatty acids (ie. hyperlipidemia) (Randle, 1989; Randle, 1963). The higher dependency on fatty acid oxidation for mitochondrial metabolism causes pronounced decreases in pyruvate dehydrogenase activity, the first irreversible reaction in the mitochondrial oxidation of glucose, due to increase in acetyl-CoA production. In addition, reduced phosphofructokinase activity, the rate-limiting step in glycolysis, is also evident due to accumulating citrate (Randle, 1989) and fructose 2,6 bisphosphate levels (French 1988). Together, the evidence on reduced glycolytic rate with increased fatty acid oxidation is rather convincing from the human and experimental diabetic models.

Although glucose uptake and glycolytic rate in the diabetic myocardium is reduced, surprisingly, glycogen accumulation has been reported in human (Sakakibara *et al.*, 2011; Warren, 1930) and rodent diabetic models (Alfarano *et al.*, 2011; Lajoie *et al.*, 2004; Shearer *et al.*, 2011). The basis for this paradoxical glycogen accumulation in

congruence with restricted cardiac glucose supply is not clear. Evidence of sex differences from other non-pathological metabolic stress (48 hrs fasting) shows female-specific cardiac glycogen accumulation associated with differential phagic processing of glycogen (Reichelt *et al.*, 2013a). An increased dependency and reduced utilisation of glucose in ageing female, but not male hearts, was shown through FDG–PET/CT imaging in healthy human subjects (Kakinuma *et al.*, 2013). Together, these suggest sex differences in cardiac glycogen and glucose handling in diabetes.

1.5B Glycogen flux pathways in the heart

Two distinct subcellular pools of cardiac glycogen have been identified by electron microscopy: free cytosolic glycogen and glycogen enclosed in double-membrane-bound structures likely to be autophagosomes. Free cytosolic glycogen may play a role in maintaining basal intracellular glucose levels, while membrane-bound glycogen destined for glycophagic breakdown of bulk glycogen may play a role in rapid release of glucose in response to a large increase in demand. Glycogen localised to autophagosomes has been reported to be particularly prominent in neonatal hearts and some forms of GSD (Fayssol, 2008). The manner in which localisation of glycogen shifts between subsarcolemmal and intra-fibrillar sites in pathophysiologic states and the conditions that regulate particle flux via lysosomal breakdown have not yet been determined. Interestingly, distinct separation of enzymes mediating glycogen-derived glycolytic flux, as opposed to exogenous glucose-derived glycolytic flux, is apparent (Anousis *et al.*, 2004), suggesting differential roles for metabolic fuel from these sources. In the myocardium these aspects of glycogen dynamics may be particularly important in functional integrity.

Cytosolic enzymatic-regulated synthesis and breakdown of glycogen

Homeostatic glycogen metabolism is primarily mediated by enzymes localised to the glycogen particle. Upon entering the cell, glucose is phosphorylated to glucose-6-phosphate (G6P) by glucokinase. Glucose-6-phosphate can enter the glycolytic pathway via conversion to fructose-6-phosphate or glucose-1-phosphate and then to uridine diphosphate-glucose, to provide substrate for glycogen synthesis. Glycogenin catalyses the first step in glycogen synthesis by covalently attaching glucose to itself. The first few glucose residues of the glycogen strand are assembled by glycogenin and provide a primer for glycogen synthase to continue transferring uridine diphosphate-glucose

residues onto this polymeric glycogen strand via α -1,4-glycosidic bonds and to release uridine diphosphate (Roach *et al.*, 2012). After 10–14 glucose residues (the precise number remains under debate) (Roach *et al.*, 2012), the glycogen-branching enzyme attaches a strand of approximately seven glucose residues via an α -1,6-glycosidic bond. Glycogen synthase extends this chain until the glycogen-branching enzyme adds an additional branch point. This process continues, resulting in a highly branched glycogen structure. Branching acts to increase the number of terminal glucose residues available for release and increases solubility. The physiologic importance of this branching morphology is apparent in genetic conditions in which genetic glycogen-branching enzyme deficiency leads to an accumulation of insoluble glycogen (polyglucosan) with long outer branches and muscle pathology (type IV GSD) (Nase *et al.*, 1995).

The breakdown of glycogen is mediated by glycogen phosphorylase, which cleaves α -1,4-glycosidic bonds to release glucose-1-phosphate. The action of glycogen phosphorylase is limited to four residues from the glycogen branch point, which are referred to as limit dextrins (Larner *et al.*, 1952). Disassembly of the branch points requires the dual-acting glycogen-debranching enzyme (GDE) to first transfer the three terminal residues to another linear strand (via its transferase activity) and then cleave the α -1,6-glycosidic bond (via its glucosidase activity) to liberate one free glucose and release the limit dextrins for phosphorylase action (Figure 1.1) (Roach *et al.*, 2012). Cori's or Forbes disease (type III GSD) is characterised by GDE deficiency, where short glycogen strands accumulate in tissues and systemic hypoglycaemia is evident (Shen *et al.*, 2002).

Proteomic analysis of the glycogen particle has identified that all of these enzymes (with the exception of GDE) are associated with glycogen (Stapleton *et al.*, 2010). Low levels of phosphate in the glycogen particle have been detected, consistent with phosphorylation of the glycogen molecule (Tagliabracci *et al.*, 2011). The mechanism by which phosphate is incorporated into glycogen is under debate. Laforin has been identified as the phosphatase that dephosphorylates glycogen, and its expression is crucial for normal glycogen structure (Tagliabracci *et al.*, 2007). Individuals with Lafora disease exhibit mutations in laforin and present with hyperphosphorylated glycogen particles, which are poorly branched and lack solubility (Tagliabracci *et al.*, 2008). Although there have been significant recent advances in the understanding of functional branching and debranching dynamics in relation to glycogen management,

major challenges still exist with regard to determining the subtle tissue specificity of these enzymatic processes and, most particularly, to evaluating how branching disturbance contributes to myocardial glycogen usage dysfunction.

Lysosomal glycogen breakdown in the heart

A second pathway for glycogenolysis exists via phagic recruitment and lysosomal degradation, termed “glycophagy” (i.e. glycogen-specific autophagy) (Reichelt *et al.*, 2013b). Electron micrographs identify glycogen enclosed in double-membrane bound structures (likely glycophagosomes) in neonatal cardiomyocytes (Kondomerkos *et al.*, 2005). It has been recently demonstrated that a specific glycophagy pathway is operational in the adult heart (Mellor *et al.*, 2014; Reichelt *et al.*, 2013b). Proteins involved in recruiting glycogen to the forming phagosome have been identified. Starch-binding domain-containing protein 1 is involved in binding glycogen and mediating autophagosome anchorage via its Atg8-interacting motif (AIM), which appears to be specific to the Atg8 protein, GABA_A receptor-associated protein-like 1, and a homologue of the phagosomal protein light-chain 3 (Jiang *et al.*, 2010; Jiang *et al.*, 2011; Stapleton *et al.*, 2010). We have been able to demonstrate that starch-binding domain-containing protein 1 (STBD1) and light-chain 3B have discrete phagosomal immunolocalisation patterns in cardiomyocytes, indicating that autophagic trafficking of glycogen and protein cargo in cardiomyocytes can occur via distinct pathways (Figure 1.2) (Mellor *et al.*, 2014). STBD1 has recently been shown to interact with glycogen-associated proteins, such as glycogen synthase, debranching enzyme, and laforin (Zhu *et al.*, 2014).

In the lysosome, acid α -glucosidase (GAA) cleaves both the α -1,4-glycosidic bonds (without restriction by the limit dextrin) and the α -1,6-glycosidic bonds (branch points), resulting in complete degradation of the glycogen particle to free α -glucose (Onodera *et al.*, 1994; Palmer, 1971; Tashiro *et al.*, 1986). Analysis of purified GAA from the human heart has demonstrated similar substrate specificity and kinetics relative to liver GAA (Chambers *et al.*, 1983). Our recent studies have demonstrated that cardiac glycophagy and glycogen content are simultaneously upregulated in response to fasting and insulin exposure (Mellor *et al.*, 2014), suggesting that phagic glycogen breakdown may be triggered by glycogen excess. Interestingly, sex differences in glycophagy were evident under basal conditions, and women appear particularly susceptible to glycogen

and glycophagy increase in response to fasting (Reichelt *et al.*, 2013b). Further work is required to delineate the conditions that selectively promote glycophagic activity and to understand how phagic and non-phagic glycogen breakdown processes can operate in complementarity.

Despite multiple observations of cardiac glycogen accumulation in diabetes, mechanistic understanding of this pathological adaptation has not been progressed. The importance of glycogen breakdown can be evidenced in cardiac glycogen storage cardiac pathologies, which occur with mutations in those proteins that mediate the final stages of glycogen degradation. A few examples of these GSDs have been highlighted in this review.

1.5C Cardiac glycogen toxicity: Perspectives from glycogen storage diseases

Lysosomal GAA is the enzyme that catalyses the complete breakdown of glycogen to free α -glucose via lysosome degradation, thus constituting the final stage of the recently recognised process of glycogen autophagy. Deficiency of GAA results in Pompe's disease where lysosomal glycogen accumulation and formation of excessive vesicular structures occurs (presumably arrested glycogen phagosomes) in skeletal, cardiac, and smooth muscles (Kishnani *et al.*, 2006; Lim *et al.*, 2014). Enzyme replacement therapy (ERT) with recombinant human GAA has been shown to attenuate glycogen accumulation, cardiac hypertrophy, and conductance abnormality in the hearts of GAA-deficient mice (Mah *et al.*, 2007; Xu *et al.*, 2005; Zhu *et al.*, 2009). The phenotype of GAA deficiency demonstrates that lysosomal GAA-mediated glycogen breakdown is essential to maintain normal glycogen content in the heart.

Danon's disease, with mutations in LAMP2, depict cardiac glycogen pathology similar to that of Pompe's disease in the absence of GAA deficiency (Danon *et al.*, 1981). The cardiac phenotype involves hypertrophic cardiomyopathy, with Wolff–Parkinson–White syndrome's ECG pre-excitation pattern (Boucek *et al.*, 2011; Bui *et al.*, 2008; Yang *et al.*, 2007). Danon disease has also been classed as a subtype of autophagic vacuolar myopathies because of the presence of glycogen-filled intracellular autophagic vacuoles in human skeletal and cardiac myocytes (Nishino *et al.*, 2000; Yang *et al.*, 2007). The symptoms of Danon disease present ~15 years later in women than in men and with less severe cardiac complications, which may be attributed to a gene dosage effect because LAMP2 is located on the X chromosome (Arad *et al.*, 2005). Thus, the cardiac

phenotype related to LAMP2 deficiency further validates an important role for lysosomal breakdown of glycogen in maintaining glycogen content at a sub-pathological level in the heart.

Mutation in the glycogen-debranching enzyme gene (gene name: *AGL*) results in an accumulation of an intermediate form of glycogen with short branches of four glucose residues, the limit dextrins, in the heart (Austin *et al.*, 2012). The clinical manifestations of GDE deficiency include cardiac fibrosis, severe vacuolation of cardiac myocytes, and glycogen accumulation in the atrioventricular node and smooth muscle cells of intramyocardial arteries (Austin *et al.*, 2012). Echocardiography analysis has reported left ventricular hypertrophy and diastolic dysfunction with preserved systolic function (Olson *et al.*, 1984). Recombinant human GAA has also effectively reduced glycogen levels in skeletal muscles of GSD III individuals, suggesting that upregulation of the lysosomal glycogen breakdown pathway may bypass the GDE obstruction in the phosphorylase-glycogen breakdown route (Sun *et al.*, 2013). Intact GDE activity appears crucial for normal glycogen breakdown in the heart, and the presence of cardiomyocyte vacuoles in tissues of GDE-deficient individuals suggests that when the glycogen breakdown route via phosphorylase is impaired, the phagic-lysosomal route of glycogen degradation is upregulated.

In summary, cardiac glycogen regulation involves a complex interplay between multiple signalling pathways, allosteric activation of enzymes and phagic degradation of glycogen. While cytosolic glycogen synthesis and breakdown are relatively well-understood, recent findings relating to phagic glycogen degradation highlight a new area of investigation in the heart. There is strong evidence from GSD that phagic/lysosomal glycogen breakdown is important for maintaining normal cardiac glycogen levels and does not simply constitute a redundant alternative breakdown route for glycogen. Advancing understanding of glycogen handling in the heart is an important priority with relevance not only to genetic GSD but also to cardiac metabolic stress disorders such as diabetes and ischemia.

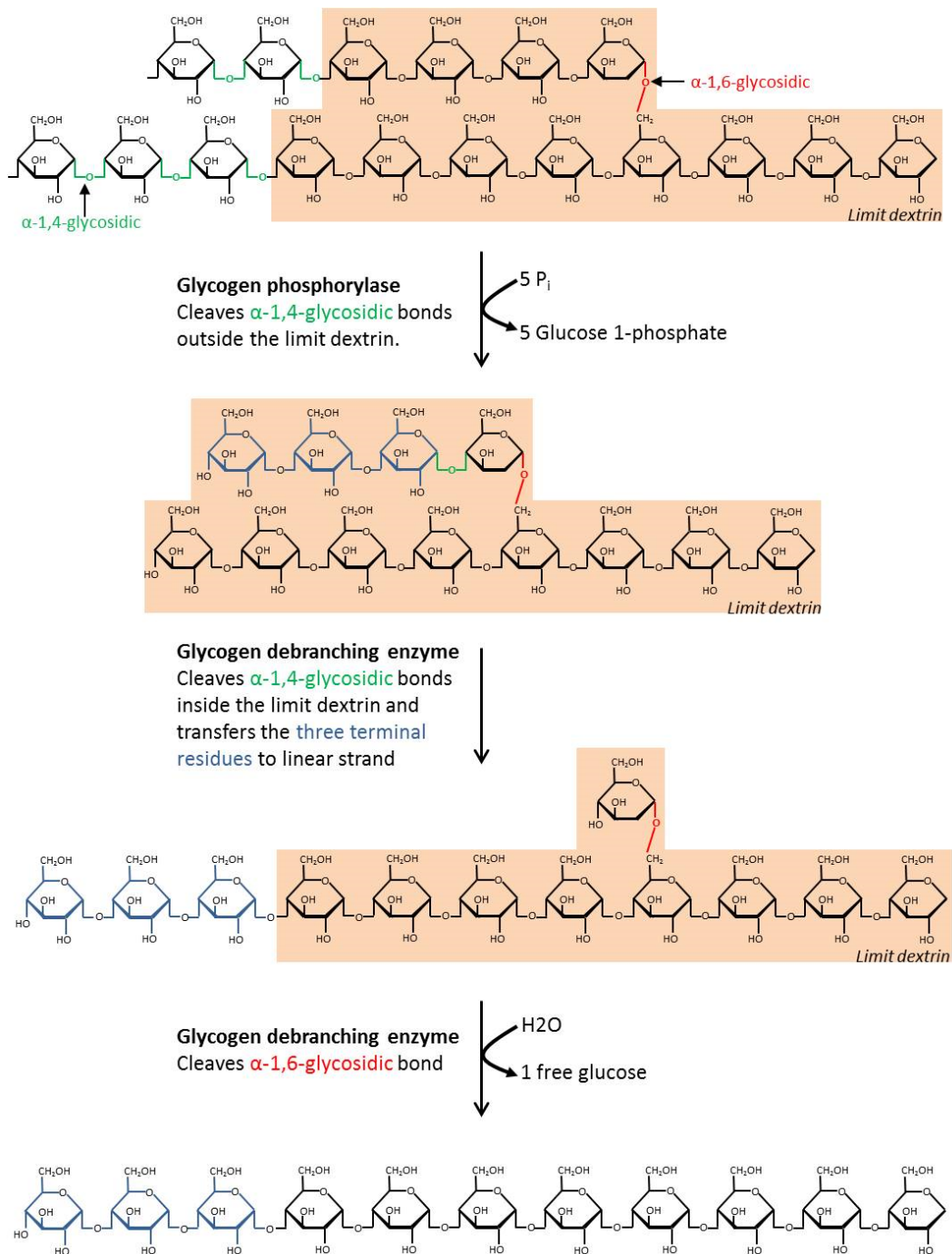


Figure 1.1. Glycogen structure and breakdown in the cytosol. Glycogen phosphorylase can cleave α -1,4-glycosidic bonds outside of the limit dextrin. Glycogen-debranching enzyme transfers the terminal 3 residues of a limit dextrin to an adjacent linear strand, then can cleave the remaining α -1,6-glycosidic branch point bond. In the lysosome these actions are done exclusively by acid- α -glucosidase.

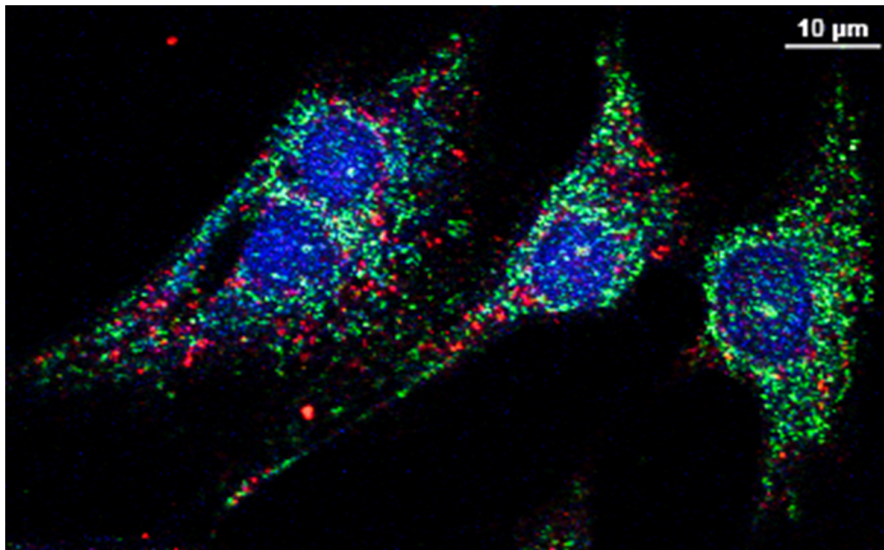


Figure 1.2. Distinct cardiomyocyte immunohistologic localisation of glycogen autophagic trafficking cargo in vitro. STBD1 (green) and LC3B (red) do not co-localise in neonatal ventricular rat myocytes. Confocal image, x63 mag: DAPI (blue) shows nuclei.

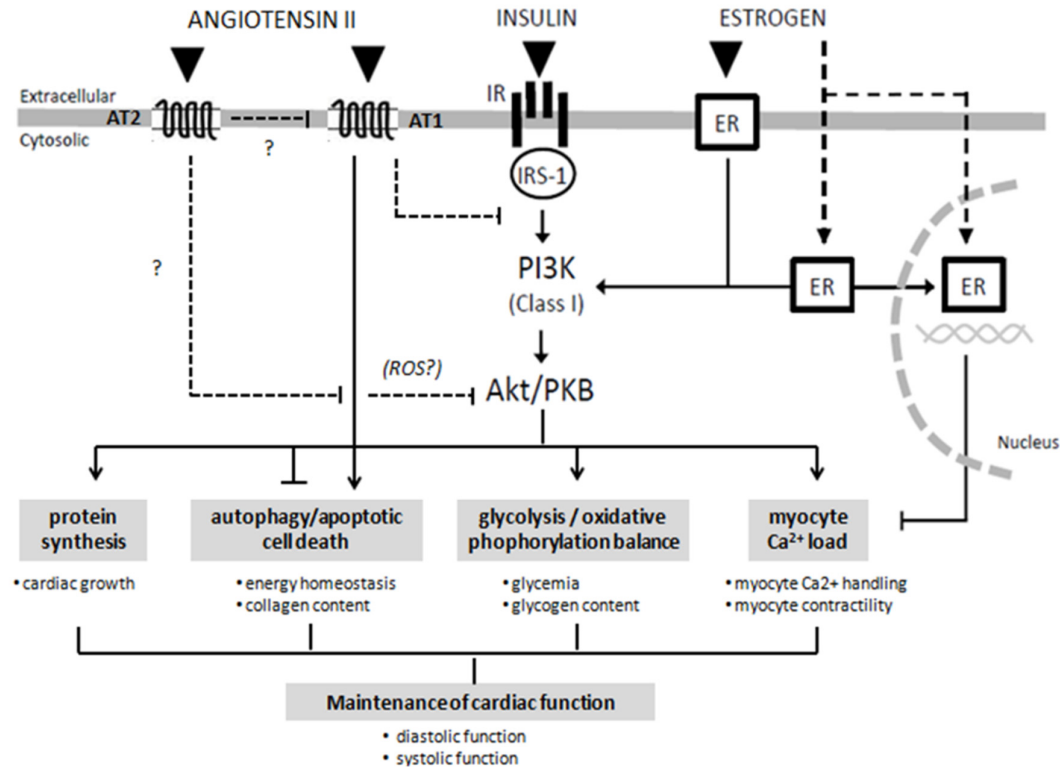


Figure 1.3. Hypothesis for interactions between insulin, AngII and estrogen signalling pathways. IRS1 upregulates Class 1 P13K and results in phosphorylation and activation of Akt/PKB. Subsequent activation of multiple downstream intermediates modulates cardiac modelling and performance (highlighted in grey). Estrogen can bind to the membrane estrogen receptors (ER) receptors to regulate the P13K/Akt pathway or can bind to nuclear-localised ER to modulate transcription and expression of channels/exchangers involved in determining intracellular Ca²⁺ levels. Excessive Angiotensin II (AngII) binding to Angiotensin Receptor Type 1 (AT1) could induce autophagy directly or through upstream inhibition of P13K/Akt by potential crosstalk between reactive oxygen species (ROS) and mitogen-activated protein kinase (MAPK) pathway. AngII binding to AT2 may oppose the action of AT1 and could potentially rescue myocytes from AT1-mediated autophagy assault. IRS1, insulin receptor substrate 1; P13K phosphoinositide-3-kinase; PKB, Protein Kinase B; ER, estrogen receptor; AngII, AngiotensinII; AT1, Angiotensin Receptor Type 1; AT2, Angiotensin Receptor Type 2.

1.6 Diabetes, sex and AngII in the heart – new questions

Diabetic cardiomyopathy is characterised by diastolic dysfunction, increased fibrosis, metabolic perturbations and signalling abnormalities. The Framingham study provided the first evidence that cardiovascular risk is elevated for all diabetics and females are particularly vulnerable. Numerous experimental diabetic models have been developed and recapitulate to varying extents the diastolic and systolic dysfunction observed in human diabetic patients. Perplexingly, the importance of sexual dimorphism in diabetic cardiomyopathy has not yet been explored leading to a lack sex-specific mechanistic insights.

Clinical observations of increased occurrence of acute myocardial infarction in women have not been extensively explored experimentally. Paradoxically, many *in vivo* and *ex vivo* T1D and T2D experimental ischemia-reperfusion models show less sensitivity to ischemic injury in diabetic hearts following acute reperfusion. A possible explanation could be a higher reliance on glycogen as an “emergency” substrate in the diabetic myocardium.

Indeed, paradoxical glycogen accumulation in the diabetic hearts (pre-ischemic) have been demonstrated in several diabetic settings although insulin-dependent glucose uptake is significantly reduced. The basis for this glycogen “defence” response is not understood. Evidence drawn from cardiac glycogen storage diseases point towards disturbances in conventional or glycophagic breakdown processes. Recently, glycophagy has been shown to exist in the heart and it can be differently regulated in males and females. Interestingly, GABARAPL1 (the protein which transfers glycogen to the phagosome) has been found to be regulated by estrogen, although in non-cardiac tissues to date. Whether glycogen plays a role in the sex-specific diabetic cardiomyopathy phenotype has not been previously examined.

Upregulated systemic and cardiac RAS is often observed as a coincidental occurrence in diabetes. AngII stimulates a myocardial fibrotic response and causes cardiac dysfunction. Recent studies have also implicated AngII in macro-autophagy in the heart. Therefore, this suggests a more important role of RAS in the diabetic myocardium. The possible crosstalk between the AngII and insulin signalling pathway is yet to be fully elucidated. Suppressed insulin signalling in combination with elevated AngII could potentially augment autophagy in the heart. The significant myocyte loss in diabetes

which follows may result in marked pathological consequences and confer greater susceptibility to acute ischemic stress. Additionally, a local estrogen regulation of AngII in the heart is possible due to the estrogen-response element found in the angiotensinogen gene. However, sex-specific effects in experimental diabetic models with angiotensin intervention have not been examined.

Therefore, the goal of this project is to evaluate sex differences in cellular mechanisms of diastolic dysfunction associated with cardiac glucose handling abnormalities. This thesis has predominantly focused on using an STZ-induced T1D setting. Novel findings have further directed investigations in other rodent T1D and T2D models. The four experimental questions addressing these knowledge deficits are:

1. Are systemic and cardiac diabetic phenotypes similar in males and females?
2. Is there an accentuated female vulnerability to ischemia in diabetes?
3. Are cell death and metabolism pathways different in both sexes with diabetes?
4. Does cardiac RAS upregulation interact with sex-specific responses in diabetes?

A wide range of *in vivo* and *ex vivo* strategies have been employed to address these broad questions. Experimental investigations include functional, structural, molecular and histological characterisations of T1D at cardiac and systemic levels, and some molecular exploration in other diabetic models. The general methodology has been detailed in Chapter 2, with specific methods relating to each study outlined in the respective experimental chapters.

1.7 References

Abel ED, Kaulbach HC, Tian R, Hopkins JC, Duffy J, Doetschman T, Minnemann T, Boers ME, Hadro E, Oberste-Berghaus C, Quist W, Lowell BB, Ingwall JS, and Kahn BB. Cardiac hypertrophy with preserved contractile function after selective deletion of glut4 from the heart. *J Clin Invest* 1999 **104**: 1703-1714.

Agvall B, and Dahlstrom U. Patients in primary health care diagnosed and treated as heart failure, with special reference to gender differences. *Scand J Prim Health Care* 2001 **19**: 14-19.

Alfarano C, Suffredini S, Fantappie O, Mugelli A, Cerbai E, Manni ME, and Raimondi L. The effect of losartan treatment on the response of diabetic cardiomyocytes to atp depletion. *Pharmacol Res* 2011 **63**: 225-232.

Annapurna A, Reddy CS, Akondi RB, and Rao SR. Cardioprotective actions of two bioflavonoids, quercetin and rutin, in experimental myocardial infarction in both normal and streptozotocin-induced type i diabetic rats. *J Pharm Pharmacol* 2009 **61**: 1365-1374.

Anousis N, Carvalho RA, Zhao P, Malloy CR, and Sherry AD. Compartmentation of glycolysis and glycogenolysis in the perfused rat heart. *NMR Biomed* 2004 **17**: 51-59.

Arad M, Maron BJ, Gorham JM, Johnson WH, Jr., Saul JP, Perez-Atayde AR, Spirito P, Wright GB, Kanter RJ, Seidman CE, and Seidman JG. Glycogen storage diseases presenting as hypertrophic cardiomyopathy. *N Engl J Med* 2005 **352**: 362-372.

Austin SL, Proia AD, Spencer-Manzon MJ, Butany J, Wechsler SB, and Kishnani PS. Cardiac pathology in glycogen storage disease type iii. *JIMD Rep* 2012 **6**: 65-72.

Barouch LA, Gao D, Chen L, Miller KL, Xu W, Phan AC, Kittleson MM, Minhas KM, Berkowitz DE, Wei C, and Hare JM. Cardiac myocyte apoptosis is associated with increased DNA damage and decreased survival in murine models of obesity. *Circ Res* 2006 **98**: 119-124.

Barrett-Connor EL, Cohn BA, Wingard DL, and Edelstein SL. Why is diabetes mellitus a stronger risk factor for fatal ischemic heart disease in women than in men? The rancho bernardo study. *JAMA* 1991 **265**: 627-631.

Basu R, Dalla Man C, Campioni M, Basu A, Klee G, Toffolo G, Cobelli C, and Rizza RA. Effects of age and sex on postprandial glucose metabolism: Differences in

glucose turnover, insulin secretion, insulin action, and hepatic insulin extraction. *Diabetes* 2006 **55**: 2001-2014.

Baynes JW, and Murray DB. The metal chelators, trientine and citrate, inhibit the development of cardiac pathology in the zucker diabetic rat. *Exp Diabetes Res* 2009 **2009**: 696378.

Belke DD, Swanson EA, and Dillmann WH. Decreased sarcoplasmic reticulum activity and contractility in diabetic db/db mouse heart. *Diabetes* 2004 **53**: 3201-3208.

Benazzoug Y, Borchellini C, Labat-Robert J, Robert L, and Kern P. Effect of high-glucose concentrations on the expression of collagens and fibronectin by fibroblasts in culture. *Exp Gerontol* 1998 **33**: 445-455.

Besford QA, Sullivan MA, Zheng L, Gilbert RG, Stapleton D, and Gray-Weale A. The structure of cardiac glycogen in healthy mice. *Int J Biol Macromol* 2012 **51**: 887-891.

Bollano E, Omerovic E, Svensson H, Waagstein F, and Fu M. Cardiac remodeling rather than disturbed myocardial energy metabolism is associated with cardiac dysfunction in diabetic rats. *Int J Cardiol* 2007 **114**: 195-201.

Boonman-de Winter LJ, Cramer MJ, Hoes AW, and Rutten FH. Uncovering heart failure with preserved ejection fraction in patients with type 2 diabetes in primary care: Time for a change. *Neth Heart J* 2016 **24**: 237-243.

Borissova AM, Tankova T, Kirilov G, and Koev D. Gender-dependent effect of ageing on peripheral insulin action. *Int J Clin Pract* 2005 **59**: 422-426.

Boucek D, Jirikowic J, and Taylor M. Natural history of danon disease. *Genet Med* 2011 **13**: 563-568.

Boudina S, and Abel ED. Diabetic cardiomyopathy revisited. *Circulation* 2007 **115**: 3213-3223.

Boyer JK, Thanigaraj S, Schechtman KB, and Perez JE. Prevalence of ventricular diastolic dysfunction in asymptomatic, normotensive patients with diabetes mellitus. *Am J Cardiol* 2004 **93**: 870-875.

Brenner BM, Cooper ME, de Zeeuw D, Keane WF, Mitch WE, Parving HH, Remuzzi G, Snapinn SM, Zhang Z, and Shahinfar S. Effects of losartan on renal and

cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 2001 **345**: 861-869.

Bui YK, Renella P, Martínez-Agosto JA, Verity A, Madikians A, and Alejos JC. Danon disease with typical early-onset cardiomyopathy in a male: Focus on a novel lamp-2 mutation. *Pediatr Transplant* 2008 **12**: 246-250.

Cárdenas G, Carlos Torres J, Zamora J, and Baños G. Isolated heart function during ischemia and reperfusion in sucrose-fed rats: Effect of insulin infusion. *Cardiovasc Pathol* 2005 **14**: 256-264.

Cárdenas G, Carlos Torres J, Zamora J, Pérez I, and Baños G. Isolated heart function after ischemia and reperfusion in sucrose-fed rats: Influence of gender and treatment. *Clin Exp Hypertens* 2006 **28**: 85-107.

Carley AN, Semeniuk LM, Shimoni Y, Aasum E, Larsen TS, Berger JP, and Severson DL. Treatment of type 2 diabetic db/db mice with a novel ppargamma agonist improves cardiac metabolism but not contractile function. *Am J Physiol Endocrinol Metab* 2004 **286**: E449-455.

Carpiuc KT, Wingard DL, Kritz-Silverstein D, and Barrett-Connor E. The association of angina pectoris with heart disease mortality among men and women by diabetes status: The rancho bernardo study. *J Womens Health (Larchmt)* 2010 **19**: 1433-1439.

Ceylan-Isik AF, LaCour KH, and Ren J. Gender disparity of streptozotocin-induced intrinsic contractile dysfunction in murine ventricular myocytes: Role of chronic activation of akt. *Clin Exp Pharmacol Physiol* 2006a **33**: 102-108.

Ceylan-Isik AF, LaCour KH, and Ren J. Sex difference in cardiomyocyte function in normal and metallothionein transgenic mice: The effect of diabetes mellitus. *J Appl Physiol* 2006b **100**: 1638-1646.

Chambers JP, and Williams JC. Acid alpha-glucosidase from human heart. *Enzyme* 1983 **29**: 109-119.

Chandler MP, Morgan EE, McElfresh TA, Kung TA, Rennison JH, Hoit BD, and Young ME. Heart failure progression is accelerated following myocardial infarction in type 2 diabetic rats. *Am J Physiol Heart Circ Physiol* 2007 **293**: H1609-1616.

Chang KC, Liang JT, Tseng CD, Wu ET, Hsu KL, Wu MS, Lin YT, and Tseng YZ. Aminoguanidine prevents fructose-induced deterioration in left ventricular-arterial coupling in wistar rats. *Br J Pharmacol* 2007 **151**: 341-346.

Chen JS, Wang HJ, Chang WC, Jao CC, Wu BT, Shyu WC, and Lee SD. Altered nitroxidergic and nmda receptor-mediated modulation of baroreflex-mediated heart rate in obese zucker rats. *Hypertens Res* 2010 **33**: 932-936.

Chess DJ, Lei B, Hoit BD, Azimzadeh AM, and Stanley WC. Deleterious effects of sugar and protective effects of starch on cardiac remodeling, contractile dysfunction, and mortality in response to pressure overload. *Am J Physiol Heart Circ Physiol* 2007 **293**: H1853-1860.

Chin MH, and Goldman L. Factors contributing to the hospitalization of patients with congestive heart failure. *Am J Public Health* 1997 **87**: 643-648.

Chou AF, Wong L, Weisman CS, Chan S, Bierman AS, Correa-de-Araujo R, and Scholle SH. Gender disparities in cardiovascular disease care among commercial and medicare managed care plans. *Womens Health Issues* 2007 **17**: 139-149.

Chowdhry MF, Vohra HA, and Galinanes M. Diabetes increases apoptosis and necrosis in both ischemic and nonischemic human myocardium: Role of caspases and poly-adenosine diphosphate-ribose polymerase. *J Thorac Cardiovasc Surg* 2007 **134**: 124-131, 131 e121-123.

Christoffersen C, Bollano E, Lindegaard ML, Bartels ED, Goetze JP, Andersen CB, and Nielsen LB. Cardiac lipid accumulation associated with diastolic dysfunction in obese mice. *Endocrinology* 2003 **144**: 3483-3490.

Chu KY, Lau T, Carlsson PO, and Leung PS. Angiotensin ii type 1 receptor blockade improves beta-cell function and glucose tolerance in a mouse model of type 2 diabetes. *Diabetes* 2006 **55**: 367-374.

Connelly KA, Boyle AJ, and Kelly DJ. Angiotensin ii and the cardiac complications of diabetes mellitus. *Curr Pharm Des* 2007a **13**: 2721-2729.

Connelly KA, Kelly DJ, Zhang Y, Prior DL, Martin J, Cox AJ, Thai K, Feneley MP, Tsoporis J, White KE, Krum H, and Gilbert RE. Functional, structural and molecular aspects of diastolic heart failure in the diabetic (mren-2)27 rat. *Cardiovasc Res* 2007b **76**: 280-291.

Cooper SA, Whaley-Connell A, Habibi J, Wei Y, Lastra G, Manrique C, Stas S, and Sowers JR. Renin-angiotensin-aldosterone system and oxidative stress in cardiovascular insulin resistance. *Am J Physiol Heart Circ Physiol* 2007 **293**: H2009-2023.

Couchepin C, Le KA, Bortolotti M, da Encarnacao JA, Oboni JB, Tran C, Schneiter P, and Tappy L. Markedly blunted metabolic effects of fructose in healthy young female subjects compared with male subjects. *Diabetes Care* 2008 **31**: 1254-1256.

Crespo MJ, Marrero M, Cruz N, Quidgley J, Creagh O, Torres H, and Rivera K. Diabetes alters cardiovascular responses to anaesthetic induction agents in stz-diabetic rats. *Diab Vasc Dis Res* 2011 **8**: 299-302.

Crowley A, Menon V, Lessard D, Yarzebski J, Jackson E, Gore JM, and Goldberg RJ. Sex differences in survival after acute myocardial infarction in patients with diabetes mellitus (worchester heart attack study). *Am Heart J* 2003 **146**: 824-831.

Curl CL, Wendt IR, and Kotsanas G. Effects of gender on intracellular. *Pflugers Arch* 2001 **441**: 709-716.

Dalpiaz PL, Lamas AZ, Caliman IF, Ribeiro RF, Jr., Abreu GR, Moyses MR, Andrade TU, Gouvea SA, Alves MF, Carmona AK, and Bissoli NS. Sex hormones promote opposite effects on ace and ace2 activity, hypertrophy and cardiac contractility in spontaneously hypertensive rats. *PLoS One* 2015 **10**: e0127515.

Daniels A, Linz D, van Bilsen M, Rutten H, Sadowski T, Ruf S, Juretschke HP, Neumann-Haefelin C, Munts C, van der Vusse GJ, and van Nieuwenhoven FA. Long-term severe diabetes only leads to mild cardiac diastolic dysfunction in Zucker diabetic fatty rats. *Eur J Heart Fail* 2012 **14**: 193-201.

Daniels A, van Bilsen M, Janssen BJ, Brouns AE, Cleutjens JP, Roemen TH, Schaart G, van der Velden J, van der Vusse GJ, and van Nieuwenhoven FA. Impaired cardiac functional reserve in type 2 diabetic db/db mice is associated with metabolic, but not structural, remodelling. *Acta Physiol (Oxf)* 2011 **200**: 11-22.

Danon MJ, Oh SJ, DiMauro S, Manaligod JR, Eastwood A, Naidu S, and Schliselfeld LH. Lysosomal glycogen storage disease with normal acid maltase. *Neurology* 1981 **31**: 51-57.

Danser AH, van Kats JP, Admiraal PJ, Derkx FH, Lamers JM, Verdouw PD, Saxena PR, and Schalekamp MA. Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis. *Hypertension* 1994 **24**: 37-48.

Darmellah A, Baetz D, Prunier F, Tamareille S, Rucker-Martin C, and Feuvray D. Enhanced activity of the myocardial Na^+/H^+ exchanger contributes to left ventricular hypertrophy in the goto-kakizaki rat model of type 2 diabetes: Critical role of akt. *Diabetologia* 2007 **50**: 1335-1344.

de Gasparo M, Catt KJ, Inagami T, Wright JW, and Unger T. International union of pharmacology. Xxiii. The angiotensin ii receptors. *Pharmacol Rev* 2000 **52**: 415-472.

De Mello WC, and Danser AH. Angiotensin ii and the heart : On the intracrine renin-angiotensin system. *Hypertension* 2000 **35**: 1183-1188.

Desrois M, Clarke K, Lan C, Dalmaso C, Cole M, Portha B, Cozzone PJ, and Bernard M. Upregulation of enos and unchanged energy metabolism in increased susceptibility of the aging type 2 diabetic gk rat heart to ischemic injury. *Am J Physiol Heart Circ Physiol* 2010 **299**: H1679-1686.

Desrois M, Sidell RJ, Gauguier D, Davey CL, Radda GK, and Clarke K. Gender differences in hypertrophy, insulin resistance and ischemic injury in the aging type 2 diabetic rat heart. *J Mol Cell Cardiol* 2004 **37**: 547-555.

Devereux RB, Roman MJ, Paranicas M, O'Grady MJ, Lee ET, Welty TK, Fabsitz RR, Robbins D, Rhoades ER, and Howard BV. Impact of diabetes on cardiac structure and function: The strong heart study. *Circulation* 2000 **101**: 2271-2276.

Ding Y, Zou R, Judd RL, and Zhong J. Effects of gender difference on cardiac myocyte dysfunction in streptozotocin-induced diabetic rats. *Endocrine* 2006 **29**: 135-141.

Domenighetti AA, Boixel C, Cefai D, Abriel H, and Pedrazzini T. Chronic angiotensin ii stimulation in the heart produces an acquired long qt syndrome associated with ik1 potassium current downregulation. *J Mol Cell Cardiol* 2007 **42**: 63-70.

Domenighetti AA, Danes VR, Curl CL, Favaloro JM, Proietto J, and Delbridge LM. Targeted glut-4 deficiency in the heart induces cardiomyocyte hypertrophy and impaired contractility linked with Ca^{2+} and proton flux dysregulation. *J Mol Cell Cardiol* 2010 **48**: 663-672.

Domenighetti AA, Wang Q, Egger M, Richards SM, Pedrazzini T, and Delbridge LM. Angiotensin ii-mediated phenotypic cardiomyocyte remodeling leads to age-dependent cardiac dysfunction and failure. *Hypertension* 2005 **46**: 426-432.

Dong F, and Ren J. Fildarestat improves cardiomyocyte contractile function in db/db diabetic obese mice through a histone deacetylase sir2-dependent mechanism. *J Hypertens* 2007 **25**: 2138-2147.

Dong F, Zhang X, Yang X, Esberg LB, Yang H, Zhang Z, Culver B, and Ren J. Impaired cardiac contractile function in ventricular myocytes from leptin-deficient ob/ob obese mice. *J Endocrinol* 2006 **188**: 25-36.

Dotevall A, Rosengren A, Bartnik M, Malmberg K, Ohrvik J, Simoons M, and Ryden L. Sex-related aspects on abnormal glucose regulation in patients with coronary artery disease. *Eur Heart J* 2007 **28**: 310-315.

Drochmans P. Morphologie du glycogène: Etude au microscope électronique de colorations négatives du glycogène particulaire. *J Ultrastruct Res* 1962 **6**: 141-163.

Dzau VJ. Local expression and pathophysiological role of renin-angiotensin in the blood vessels and heart. *Basic Res Cardiol* 1993 **88 Suppl 1**: 1-14.

ECDCDM. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 2003 **26 Suppl 1**: S5-20.

Editorial. Cardiovascular disease in women--often silent and fatal. *Lancet* 2011 **378**: 200.

El Dayem SM, and Battah AA. Effect of glycemic control on the progress of left ventricular hypertrophy and diastolic dysfunction in children with type i diabetes mellitus. *Anadolu Kardiyol Derg* 2012 **12**: 498-507.

Engeli S, Negrel R, and Sharma AM. Physiology and pathophysiology of the adipose tissue renin-angiotensin system. *Hypertension* 2000 **35**: 1270-1277.

Fang ZY, Schull-Meade R, Downey M, Prins J, and Marwick TH. Determinants of subclinical diabetic heart disease. *Diabetologia* 2005 **48**: 394-402.

Fayssol A. Cardiomyopathy in pompe's disease. *Eur J Intern Med* 2008 **19**: 57-59.

Feldmer M, Kaling M, Takahashi S, Mullins JJ, and Ganten D. Glucocorticoid- and estrogen-responsive elements in the 5'-flanking region of the rat angiotensinogen gene. *J Hypertens* 1991 **9**: 1005-1012.

Fiordaliso F, Li B, Latini R, Sonnenblick EH, Anversa P, Leri A, and Kajstura J. Myocyte death in streptozotocin-induced diabetes in rats in angiotensin ii- dependent. *Lab Invest* 2000 **80**: 513-527.

Flanagan DE, Holt RI, Owens PC, Cockington RJ, Moore VM, Robinson JS, Godsland IF, and Phillips DI. Gender differences in the insulin-like growth factor axis response to a glucose load. *Acta Physiol (Oxf)* 2006 **187**: 371-378.

Folsom AR, Szklo M, Stevens J, Liao F, Smith R, and Eckfeldt JH. A prospective study of coronary heart disease in relation to fasting insulin, glucose, and diabetes. The atherosclerosis risk in communities (aric) study. *Diabetes Care* 1997 **20**: 935-942.

Fraser GE, Strahan TM, Sabate J, Beeson WL, and Kissinger D. Effects of traditional coronary risk factors on rates of incident coronary events in a low-risk population. The adventist health study. *Circulation* 1992 **86**: 406-413.

Fredersdorf S, Thumann C, Ulucan C, Griese DP, Luchner A, Riegger GA, Kromer EP, and Weil J. Myocardial hypertrophy and enhanced left ventricular contractility in zucker diabetic fatty rats. *Cardiovasc Pathol* 2004 **13**: 11-19.

Frustaci A, Kajstura J, Chimenti C, Jakoniuk I, Leri A, Maseri A, Nadal-Ginard B, and Anversa P. Myocardial cell death in human diabetes. *Circulation research* 2000 **87**: 1123-1132.

Furman BL. Streptozotocin-induced diabetic models in mice and rats. *Curr Protoc Pharmacol* 2015 **70**: 5.47.41-20.

Galderisi M. Diastolic dysfunction and diabetic cardiomyopathy: Evaluation by doppler echocardiography. *J Am Coll Cardiol* 2006 **48**: 1548-1551.

Galipeau D, Verma S, and McNeill JH. Female rats are protected against fructose-induced changes in metabolism and blood pressure. *Am J Physiol Heart Circ Physiol* 2002a **283**: H2478-2484.

Galipeau DM, Yao L, and McNeill JH. Relationship among hyperinsulinemia, insulin resistance, and hypertension is dependent on sex. *Am J Physiol Heart Circ Physiol* 2002b **283**: H562-567.

Galvan AQ, Galetta F, Natali A, Muscelli E, Sironi AM, Cini G, Camastra S, and Ferrannini E. Insulin resistance and hyperinsulinemia: No independent relation to left ventricular mass in humans. *Circulation* 2000 **102**: 2233-2238.

Ganten D, Marquez-Julio A, Granger P, Hayduk K, Karsunky K, Boucher R, and Genest J. Renin in dog brain. *Am J Physiol* 1971 **221**: 1733-1737.

Gao F, Gao E, Yue TL, Ohlstein EH, Lopez BL, Christopher TA, and Ma XL. Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: The roles of pi3-kinase, akt, and endothelial nitric oxide synthase phosphorylation. *Circulation* 2002 **105**: 1497-1502.

Ge F, Hu C, Hyodo E, Arai K, Zhou S, Lobdell Iv H, Walewski JL, Homma S, and Berk PD. Cardiomyocyte triglyceride accumulation and reduced ventricular function in mice with obesity reflect increased long chain fatty acid uptake and de novo fatty acid synthesis. *Journal of obesity* 2012 **2012**: 205648.

Gladden JD, Linke WA, and Redfield MM. Heart failure with preserved ejection fraction. *Pflugers Arch* 2014 **466**: 1037-1053.

Glumer C, Jorgensen T, and Borch-Johnsen K. Prevalences of diabetes and impaired glucose regulation in a danish population: The inter99 study. *Diabetes Care* 2003 **26**: 2335-2340.

Grundey SM, Benjamin IJ, Burke GL, Chait A, Eckel RH, Howard BV, Mitch W, Smith SC, Jr., and Sowers JR. Diabetes and cardiovascular disease: A statement for healthcare professionals from the american heart association. *Circulation* 1999 **100**: 1134-1146.

Hadour G, Ferrera R, Sebbag L, Forrat R, Delaye J, and de Lorgeril M. Improved myocardial tolerance to ischaemia in the diabetic rabbit. *J Mol Cell Cardiol* 1998 **30**: 1869-1875.

Hafstad AD, Khalid AM, How OJ, Larsen TS, and Aasum E. Glucose and insulin improve cardiac efficiency and postischemic functional recovery in perfused hearts from type 2 diabetic (db/db) mice. *Am J Physiol Endocrinol Metab* 2007 **292**: E1288-1294.

Hafstad AD, Solevag GH, Severson DL, Larsen TS, and Aasum E. Perfused hearts from type 2 diabetic (db/db) mice show metabolic responsiveness to insulin. *Am J Physiol Heart Circ Physiol* 2006 **290**: H1763-1769.

Hayes AJ, Leal J, Kelman CW, and Clarke PM. Risk equations to predict life expectancy of people with type 2 diabetes mellitus following major complications: A study from western australia. *Diabet Med* 2011a **28**: 428-435.

Hayes SN, Wenger NK, Greenberger MSW, and M. TL. 10q report: Advancing women's heart health through improved research, diagnosis and treatment 2011b.

Heyden S, Heiss G, Bartel AG, and Hames CG. Sex differences in coronary mortality among diabetics in evans county, georgia. *J Chronic Dis* 1980 **33**: 265-273.

Ho CY, and Solomon SD. A clinician's guide to tissue doppler imaging. *Circulation* 2006 **113**: e396-398.

Hofmann PA, Menon V, and Gannaway KF. Effects of diabetes on isometric tension as a function of $[Ca^{2+}]$ and pH in rat skinned cardiac myocytes. *Am J Physiol* 1995 **269**: H1656-1663.

Hoit BD, Castro C, Bultron G, Knight S, and Matlib MA. Noninvasive evaluation of cardiac dysfunction by echocardiography in streptozotocin-induced diabetic rats. *J Card Fail* 1999 **5**: 324-333.

Horton TJ, Gayles EC, Prach PA, Koppenhafer TA, and Pagliassotti MJ. Female rats do not develop sucrose-induced insulin resistance. *The American journal of physiology* 1997 **272**: R1571-1576.

Howarth FC, Qureshi A, Shahin A, and Lukic ML. Effects of single high-dose and multiple low-dose streptozotocin on contraction and intracellular Ca^{2+} in ventricular myocytes from diabetes resistant and susceptible rats. *Mol Cell Biochem* 2005 **269**: 103-108.

Howarth FC, and Qureshi MA. Myofilament sensitivity to Ca^{2+} in ventricular myocytes from the goto-kakizaki diabetic rat. *Mol Cell Biochem* 2008 **315**: 69-74.

Howarth FC, Qureshi MA, Hassan Z, Isaev D, Parekh K, John A, Oz M, Raza H, Adeghate E, and Adrian TE. Contractility of ventricular myocytes is well preserved despite altered mechanisms of Ca^{2+} transport and a changing pattern of mRNA in aged type 2 zucker diabetic fatty rat heart. *Mol Cell Biochem* 2012 **361**: 267-280.

Hu ZC, Chen YD, and Ren YH. Methylprednisolone improves microcirculation in streptozotocin-induced diabetic rats after myocardial ischemia/reperfusion. *Chin Med J (Engl)* 2011 **124**: 923-929.

Huggins CE, Domenighetti AA, Ritchie ME, Khalil N, Favaloro JM, Proietto J, Smyth GK, Pepe S, and Delbridge LM. Functional and metabolic remodelling in glut4-deficient hearts confers hyper-responsiveness to substrate intervention. *J Mol Cell Cardiol* 2008 **44**: 270-280.

Huxley R, Barzi F, and Woodward M. Excess risk of fatal coronary heart disease associated with diabetes in men and women: Meta-analysis of 37 prospective cohort studies. *BMJ* 2006 **332**: 73-78.

Huynh K, Kiriazis H, Du XJ, Love JE, Jandeleit-Dahm KA, Forbes JM, McMullen JR, and Ritchie RH. Coenzyme q10 attenuates diastolic dysfunction, cardiomyocyte hypertrophy and cardiac fibrosis in the db/db mouse model of type 2 diabetes. *Diabetologia* 2012 **55**: 1544-1553.

Huynh K, McMullen JR, Julius TL, Tan JW, Love JE, Cemerlang N, Kiriazis H, Du XJ, and Ritchie RH. Cardiac-specific igf-1 receptor transgenic expression protects against cardiac fibrosis and diastolic dysfunction in a mouse model of diabetic cardiomyopathy. *Diabetes* 2010 **59**: 1512-1520.

Isfort M, Stevens SC, Schaffer S, Jong CJ, and Wold LE. Metabolic dysfunction in diabetic cardiomyopathy. *Heart failure reviews* 2013

Jandeleit-Dahm KA, Tikellis C, Reid CM, Johnston CI, and Cooper ME. Why blockade of the renin-angiotensin system reduces the incidence of new-onset diabetes. *J Hypertens* 2005 **23**: 463-473.

Jiang S, Heller B, Tagliabracci VS, Zhai L, Irimia JM, DePaoli-Roach AA, Wells CD, Skurat AV, and Roach PJ. Starch binding domain-containing protein 1/genethonin 1 is a novel participant in glycogen metabolism. *J Biol Chem* 2010 **285**: 34960-34971.

Jiang S, Wells CD, and Roach PJ. Starch-binding domain-containing protein 1 (stbd1) and glycogen metabolism: Identification of the atg8 family interacting motif (aim) in stbd1 required for interaction with gabarapl1. *Biochem Biophys Res Commun* 2011 **413**: 420-425.

Joffe, II, Travers KE, Perreault-Micale CL, Hampton T, Katz SE, Morgan JP, and Douglas PS. Abnormal cardiac function in the streptozotocin-induced non-insulin-

dependent diabetic rat: Noninvasive assessment with doppler echocardiography and contribution of the nitric oxide pathway. *J Am Coll Cardiol* 1999 **34**: 2111-2119.

Jones SP, Girod WG, Granger DN, Palazzo AJ, and Lefer DJ. Reperfusion injury is not affected by blockade of p-selectin in the diabetic mouse heart. *Am J Physiol* 1999 **277**: H763-769.

Juutilainen A, Kortelainen S, Lehto S, Ronnema T, Pyorala K, and Laakso M. Gender difference in the impact of type 2 diabetes on coronary heart disease risk. *Diabetes Care* 2004 **27**: 2898-2904.

Kaczmarczyk SJ, Andrikopoulos S, Favaloro J, Domenighetti AA, Dunn A, Ernst M, Grail D, Fodero-Tavoletti M, Huggins CE, Delbridge LM, Zajac JD, and Proietto J. Threshold effects of glucose transporter-4 (glut4) deficiency on cardiac glucose uptake and development of hypertrophy. *J Mol Endocrinol* 2003 **31**: 449-459.

Kakinuma Y, Okada S, Nogami M, and Kumon Y. The human female heart incorporates glucose more efficiently than the male heart. *Int J Cardiol* 2013 **168**: 2518-2521.

Kamide K, Rakugi H, Higaki J, Okamura A, Nagai M, Moriguchi K, Ohishi M, Satoh N, Tuck ML, and Ogihara T. The renin-angiotensin and adrenergic nervous system in cardiac hypertrophy in fructose-fed rats. *Am J Hypertens* 2002 **15**: 66-71.

Kannel WB, and McGee DL. Diabetes and cardiovascular disease. The framingham study. *Jama* 1979 **241**: 2035-2038.

Kappert K, Bohm M, Schmieder R, Schumacher H, Teo K, Yusuf S, Sleight P, and Unger T. Impact of sex on cardiovascular outcome in patients at high cardiovascular risk: Analysis of the telmisartan randomized assessment study in ace-intolerant subjects with cardiovascular disease (transcend) and the ongoing telmisartan alone and in combination with ramipril global end point trial (ontarget). *Circulation* 2012 **126**: 934-941.

Kato T, Yamashita T, Sekiguchi A, Tsuneda T, Sagara K, Takamura M, Kaneko S, Aizawa T, and Fu LT. Angiotensin ii type 1 receptor blocker attenuates diabetes-induced atrial structural remodeling. *J Cardiol* 2011 **58**: 131-136.

Kersten JR, Toller WG, Gross ER, Pagel PS, and Warltier DC. Diabetes abolishes ischemic preconditioning: Role of glucose, insulin, and osmolality. *Am J Physiol Heart Circ Physiol* 2000 **278**: H1218-1224.

Khalid AM, Hafstad AD, Larsen TS, Severson DL, Boardman N, Hagve M, Berge RK, and Aasum E. Cardioprotective effect of the ppar ligand tetradecylthioacetic acid in type 2 diabetic mice. *Am J Physiol Heart Circ Physiol* 2011 **300**: H2116-2122.

Khong FL, Zhang Y, Edgley AJ, Qi W, Connelly KA, Woodman OL, Krum H, and Kelly DJ. 3',4'-dihydroxyflavonol antioxidant attenuates diastolic dysfunction and cardiac remodeling in streptozotocin-induced diabetic m(ren2)27 rats. *PLoS One* 2011 **6**: e22777.

Kim SK, Zhao ZS, Lee YJ, Lee KE, Kang SM, Choi D, Lim SK, Chung N, Lee HC, and Cha BS. Left-ventricular diastolic dysfunction may be prevented by chronic treatment with ppar-alpha or -gamma agonists in a type 2 diabetic animal model. *Diabetes Metab Res Rev* 2003 **19**: 487-493.

Kishnani PS, Hwu WL, Mandel H, Nicolino M, Yong F, Corzo D, and Infantile-Onset Pompe Disease Natural History Study G. A retrospective, multinational, multicenter study on the natural history of infantile-onset pompe disease. *J Pediatr* 2006 **148**: 671-676.

Kleinman JC, Donahue RP, Harris MI, Finucane FF, Madans JH, and Brock DB. Mortality among diabetics in a national sample. *Am J Epidemiol* 1988 **128**: 389-401.

Kondomerkos DJ, Kalamidas SA, Kotoulas OB, and Hann AC. Glycogen autophagy in the liver and heart of newborn rats. The effects of glucagon, adrenalin or rapamycin. *Histol Histopathol* 2005 **20**: 689-696.

Kotsanas G, Delbridge LM, and Wendt IR. Stimulus interval-dependent differences in ca2+ transients and contractile responses of diabetic rat cardiomyocytes. *Cardiovasc Res* 2000 **46**: 450-462.

Kristiansen SB, Lofgren B, Stottrup NB, Khatir D, Nielsen-Kudsk JE, Nielsen TT, Botker HE, and Flyvbjerg A. Ischaemic preconditioning does not protect the heart in obese and lean animal models of type 2 diabetes. *Diabetologia* 2004 **47**: 1716-1721.

Krolewski AS, Kosinski EJ, Warram JH, Leland OS, Busick EJ, Asmal AC, Rand LI, Christlieb AR, Bradley RF, and Kahn CR. Magnitude and determinants of coronary artery disease in juvenile-onset, insulin-dependent diabetes mellitus. *Am J Cardiol* 1987 **59**: 750-755.

Kuperstein R, Hanly P, Niroumand M, and Sasson Z. The importance of age and obesity on the relation between diabetes and left ventricular mass. *J Am Coll Cardiol* 2001 **37**: 1957-1962.

Laing SP, Swerdlow AJ, Slater SD, Botha JL, Burden AC, Waugh NR, Smith AW, Hill RD, Bingley PJ, Patterson CC, Qiao Z, and Keen H. The british diabetic association cohort study, ii: Cause-specific mortality in patients with insulin-treated diabetes mellitus. *Diabet Med* 1999 **16**: 466-471.

Laing SP, Swerdlow AJ, Slater SD, Burden AC, Morris A, Waugh NR, Gatling W, Bingley PJ, and Patterson CC. Mortality from heart disease in a cohort of 23,000 patients with insulin-treated diabetes. *Diabetologia* 2003 **46**: 760-765.

Lajoie C, Calderone A, Trudeau F, Lavoie N, Massicotte G, Gagnon S, and Beliveau L. Exercise training attenuated the pkb and gsk-3 dephosphorylation in the myocardium of zdf rats. *J Appl Physiol* 2004 **96**: 1606-1612.

Lakshmanan AP, Harima M, Sukumaran V, Soetikno V, Thandavarayan RA, Suzuki K, Kodama M, Nagata M, Takagi R, and Watanabe K. Modulation of at-1r/ampk-mapk cascade plays crucial role for the pathogenesis of diabetic cardiomyopathy in transgenic type 2 diabetic (spontaneous diabetic torii) rats. *Biochem Pharmacol* 2012 **83**: 653-660.

Lam CS, and Little WC. Sex and cardiovascular risk: Are women advantaged or men disadvantaged? *Circulation* 2012 **126**: 913-915.

Larner J, Illingworth B, Cori GT, and Cori CF. Structure of glycogens and amylopectins. Ii. Analysis by stepwise enzymatic degradation. *J Biol Chem* 1952 **199**: 641-651.

LaRocca TJ, Fabris F, Chen J, Benhayon D, Zhang S, McCollum L, Schecter AD, Cheung JY, Sobie EA, Hajjar RJ, and Lebeche D. Na⁺/ca²⁺ exchanger-1 protects against systolic failure in the akitains2 model of diabetic cardiomyopathy via a cxcr4/nf-kappab pathway. *Am J Physiol Heart Circ Physiol* 2012 **303**: H353-367.

Lavoie JL, and Sigmund CD. Minireview: Overview of the renin-angiotensin system--an endocrine and paracrine system. *Endocrinology* 2003 **144**: 2179-2183.

Le Douairon Lahaye S, Gratas-Delamarche A, Malarde L, Zguira S, Vincent S, Lemoine Morel S, Carre F, and Rannou Bekono F. Combined insulin treatment and intense exercise training improved basal cardiac function and ca(2+)-cycling proteins expression in type 1 diabetic rats. *Appl Physiol Nutr Metab* 2012 **37**: 53-62.

Levy D, Garrison RJ, Savage DD, Kannel WB, and Castelli WP. Prognostic implications of echocardiographically determined left ventricular mass in the framingham heart study. *N Engl J Med* 1990 **322**: 1561-1566.

Lewis EJ, Hunsicker LG, Clarke WR, Berl T, Pohl MA, Lewis JB, Ritz E, Atkins RC, Rohde R, and Raz I. Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. *N Engl J Med* 2001 **345**: 851-860.

Li SY, Yang X, Ceylan-Isik AF, Du M, Sreejayan N, and Ren J. Cardiac contractile dysfunction in lep/lep obesity is accompanied by nadph oxidase activation, oxidative modification of sarco(endo)plasmic reticulum ca²⁺-atpase and myosin heavy chain isozyme switch. *Diabetologia* 2006 **49**: 1434-1446.

Li Y, Ma J, Zhu H, Singh M, Hill D, Greer PA, Arnold JM, Abel ED, and Peng T. Targeted inhibition of calpain reduces myocardial hypertrophy and fibrosis in mouse models of type 1 diabetes. *Diabetes* 2011 **60**: 2985-2994.

Lim HS, MacFadyen RJ, and Lip GY. Diabetes mellitus, the renin-angiotensin-aldosterone system, and the heart. *Arch Intern Med* 2004 **164**: 1737-1748.

Lim JA, Li L, and Raben N. Pompe disease: From pathophysiology to therapy and back again. *Front Aging Neurosci* 2014 **6**: 177.

Liu JE, Palmieri V, Roman MJ, Bella JN, Fabsitz R, Howard BV, Welty TK, Lee ET, and Devereux RB. The impact of diabetes on left ventricular filling pattern in normotensive and hypertensive adults: The strong heart study. *J Am Coll Cardiol* 2001 **37**: 1943-1949.

Louwe MC, van der Hoorn JW, van den Berg SA, Jukema JW, Romijn JA, van Dijk KW, Rensen PC, Smit JW, and Steendijk P. Gender-dependent effects of high-fat lard diet on cardiac function in c57bl/6j mice. *Appl Physiol Nutr Metab* 2012a **37**: 214-224.

Louwe MC, van der Hoorn JW, van den Berg SA, Jukema JW, Romijn JA, Willems van Dijk K, Rensen PC, Smit JW, and Steendijk P. Gender-dependent effects of high-fat lard diet on cardiac function in c57bl/6j mice. *Appl Physiol Nutr Metab* 2012b

Lundberg V, Stegmayr B, Asplund K, Eliasson M, and Huhtasaari F. Diabetes as a risk factor for myocardial infarction: Population and gender perspectives. *J Intern Med* 1997 **241**: 485-492.

Luther JM, and Brown NJ. The renin-angiotensin-aldosterone system and glucose homeostasis. *Trends Pharmacol Sci* 2011 **32**: 734-739.

Ma G, Al-Shabrawey M, Johnson JA, Datar R, Tawfik HE, Guo D, Caldwell RB, and Caldwell RW. Protection against myocardial ischemia/reperfusion injury by short-term diabetes: Enhancement of vegf formation, capillary density, and activation of cell survival signaling. *Naunyn Schmiedebergs Arch Pharmacol* 2006 **373**: 415-427.

Mah C, Pacak CA, Cresawn KO, Deruisseau LR, Germain S, Lewis MA, Cloutier DA, Fuller DD, and Byrne BJ. Physiological correction of pompe disease by systemic delivery of adeno-associated virus serotype 1 vectors. *Mol Ther* 2007 **15**: 501-507.

Marfella R, D'Amico M, Di Filippo C, Piegari E, Nappo F, Esposito K, Berrino L, Rossi F, and Giugliano D. Myocardial infarction in diabetic rats: Role of hyperglycaemia on infarct size and early expression of hypoxia-inducible factor 1. *Diabetologia* 2002 **45**: 1172-1181.

Mascareno E, Beckles D, Dhar-Mascareno M, and Siddiqui MA. Enhanced hypertrophy in ob/ob mice due to an impairment in expression of atrial natriuretic peptide. *Vascul Pharmacol* 2009 **51**: 198-204.

Matsumoto T, Kakami M, Kobayashi T, and Kamata K. Gender differences in vascular reactivity to endothelin-1 (1-31) in mesenteric arteries from diabetic mice. *Peptides* 2008 **29**: 1338-1346.

Mazzolai L, Pedrazzini T, Nicoud F, Gabbiani G, Brunner HR, and Nussberger J. Increased cardiac angiotensin ii levels induce right and left ventricular hypertrophy in normotensive mice. *Hypertension* 2000 **35**: 985-991.

Mejthert M, Holmgren J, Wandell P, Persson H, and Edner M. Diagnostic tests, treatment and follow-up in heart failure patients--is there a gender bias in the coherence to guidelines? *Eur J Heart Fail* 1999 **1**: 407-410.

Mellor KM, Bell JR, Young MJ, Ritchie RH, and Delbridge LM. Myocardial autophagy activation and suppressed survival signaling is associated with insulin resistance in fructose-fed mice. *J Mol Cell Cardiol* 2011 **50**: 1035-1043.

Mellor KM, Varma U, Stapleton D, and Delbridge LMD. Cardiomyocyte glycophagy is regulated by insulin and exposure to high extracellular glucose. *Am J Physiol Heart Circ Physiol* 2014 **306**: H1240-1245.

Meloni M, Descamps B, Caporali A, Zentilin L, Floris I, Giacca M, and Emanuelli C. Nerve growth factor gene therapy using adeno-associated viral vectors prevents cardiomyopathy in type 1 diabetic mice. *Diabetes* 2012 **61**: 229-240.

Miyazaki M, and Takai S. Tissue angiotensin ii generating system by angiotensin-converting enzyme and chymase. *J Pharmacol Sci* 2006 **100**: 391-397.

Mizushige K, Yao L, Noma T, Kiyomoto H, Yu Y, Hosomi N, Ohmori K, and Matsuo H. Alteration in left ventricular diastolic filling and accumulation of myocardial collagen at insulin-resistant prediabetic stage of a type ii diabetic rat model. *Circulation* 2000 **101**: 899-907.

Moore A, Shindikar A, Fomison-Nurse I, Riu F, Munasinghe PE, Ram TP, Saxena P, Coffey S, Bunton RW, Galvin IF, Williams MJ, Emanuelli C, Madeddu P, and Katare R. Rapid onset of cardiomyopathy in stz-induced female diabetic mice involves the downregulation of pro-survival pim-1. *Cardiovasc Diabetol* 2014 **13**: 68.

Nakayama M, Inoguchi T, Sonta T, Maeda Y, Sasaki S, Sawada F, Tsubouchi H, Sonoda N, Kobayashi K, Sumimoto H, and Nawata H. Increased expression of nad(p)h oxidase in islets of animal models of type 2 diabetes and its improvement by an at1 receptor antagonist. *Biochem Biophys Res Commun* 2005 **332**: 927-933.

Nase S, Kunze KP, Sigmund M, Schroeder JM, Shin Y, and Hanrath P. A new variant of type iv glycogenosis with primary cardiac manifestation and complete branching enzyme deficiency. In vivo detection by heart muscle biopsy. *Eur Heart J* 1995 **16**: 1698-1704.

Natarajan S, Liao Y, Cao G, Lipsitz SR, and McGee DL. Sex differences in risk for coronary heart disease mortality associated with diabetes and established coronary heart disease. *Arch Intern Med* 2003 **163**: 1735-1740.

Nawata T, Takahashi N, Ooie T, Kaneda K, Saikawa T, and Sakata T. Cardioprotection by streptozotocin-induced diabetes and insulin against ischemia/reperfusion injury in rats. *J Cardiovasc Pharmacol* 2002 **40**: 491-500.

Nielsen AH, Schauser KH, and Poulsen K. Current topic: The uteroplacental renin-angiotensin system. *Placenta* 2000 **21**: 468-477.

Nishino I, Fu J, Tanji K, Yamada T, Shimojo S, Koori T, Mora M, Riggs JE, Oh SJ, Koga Y, Sue CM, Yamamoto A, Murakami N, Shanske S, Byrne E, Bonilla E,

Nonaka I, DiMauro S, and Hirano M. Primary lamp-2 deficiency causes x-linked vacuolar cardiomyopathy and myopathy (danon disease). *Nature* 2000 **406**: 906-910.

Norhammar A, and Schenck-Gustafsson K. Type 2 diabetes and cardiovascular disease in women. *Diabetologia* 2013 **56**: 1-9.

Norhammar A, Stenestrand U, Lindback J, Wallentin L, Register of I, and Knowledge about Swedish Heart Intensive Care A. Women younger than 65 years with diabetes mellitus are a high-risk group after myocardial infarction: A report from the swedish register of information and knowledge about swedish heart intensive care admission (riks-hia). *Heart* 2008 **94**: 1565-1570.

Nuno DW, and Lamping KG. The role of rho kinase in sex-dependent vascular dysfunction in type 1 diabetes. *Experimental diabetes research* 2010 **2010**: 176361.

Oakes ND, Thalen P, Aasum E, Edgley A, Larsen T, Furler SM, Ljung B, and Severson D. Cardiac metabolism in mice: Tracer method developments and in vivo application revealing profound metabolic inflexibility in diabetes. *Am J Physiol Endocrinol Metab* 2006 **290**: E870-881.

Olivares-Reyes JA, Arellano-Plancarte A, and Castillo-Hernandez JR. Angiotensin ii and the development of insulin resistance: Implications for diabetes. *Mol Cell Endocrinol* 2009 **302**: 128-139.

Olson LJ, Reeder GS, Noller KL, Edwards WD, Howell RR, and Michels VV. Cardiac involvement in glycogen storage disease iii: Morphologic and biochemical characterization with endomyocardial biopsy. *Am J Cardiol* 1984 **53**: 980-981.

Onodera S, Matsui H, and Chiba S. Substrate specificity and subsite affinities of rabbit liver acid alpha-glucosidase. *J Biochem* 1994 **116**: 7-11.

Ooie T, Takahashi N, Nawata T, Arikawa M, Yamanaka K, Kajimoto M, Shinohara T, Shigematsu S, Hara M, Yoshimatsu H, and Saikawa T. Ischemia-induced translocation of protein kinase c-epsilon mediates cardioprotection in the streptozotocin-induced diabetic rat. *Circ J* 2003 **67**: 955-961.

Ouhoumane N, Abdous B, Emond V, and Poirier P. Impact of diabetes and gender on survival after acute myocardial infarction in the province of quebec, canada--a population-based study. *Diabet Med* 2009 **26**: 609-616.

Ouwens DM, Diamant M, Fodor M, Habets DD, Pelsers MM, El Hasnaoui M, Dang ZC, van den Brom CE, Vlasblom R, Rietdijk A, Boer C, Coort SL, Glatz JF, and Luiken JJ. Cardiac contractile dysfunction in insulin-resistant rats fed a high-fat diet is associated with elevated cd36-mediated fatty acid uptake and esterification. *Diabetologia* 2007 **50**: 1938-1948.

Palmer TN. The substrate specificity of acid -glucosidase from rabbit muscle. *Biochem J* 1971 **124**: 701-711.

Palmieri V, Capaldo B, Russo C, Iaccarino M, Pezzullo S, Quintavalle G, Di Minno G, Riccardi G, and Celentano A. Uncomplicated type 1 diabetes and preclinical left ventricular myocardial dysfunction: Insights from echocardiography and exercise cardiac performance evaluation. *Diabetes Res Clin Pract* 2008 **79**: 262-268.

Palomar-Morales M, Morimoto S, Mendoza-Rodriguez CA, and Cerbon MA. The protective effect of testosterone on streptozotocin-induced apoptosis in beta cells is sex specific. *Pancreas* 2010 **39**: 193-200.

Panagia M, Schneider JE, Brown B, Cole MA, and Clarke K. Abnormal function and glucose metabolism in the type-2 diabetic db/db mouse heart. *Can J Physiol Pharmacol* 2007 **85**: 289-294.

Park CW, Kim HW, Lim JH, Yoo KD, Chung S, Shin SJ, Chung HW, Lee SJ, Chae CB, Kim YS, and Chang YS. Vascular endothelial growth factor inhibition by drk6 causes endothelial apoptosis, fibrosis, and inflammation in the heart via the akt/enos axis in db/db mice. *Diabetes* 2009 **58**: 2666-2676.

Parving HH, Lehnert H, Brochner-Mortensen J, Gomis R, Andersen S, and Arner P. The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes. *N Engl J Med* 2001 **345**: 870-878.

Patten RD, Pourati I, Aronovitz MJ, Baur J, Celestin F, Chen X, Michael A, Haq S, Nuedling S, Grohe C, Force T, Mendelsohn ME, and Karas RH. 17beta-estradiol reduces cardiomyocyte apoptosis in vivo and in vitro via activation of phospho-inositide-3 kinase/akt signaling. *Circulation research* 2004 **95**: 692-699.

Paul M, Poyan Mehr A, and Kreutz R. Physiology of local renin-angiotensin systems. *Physiol Rev* 2006 **86**: 747-803.

Paulson DJ. The diabetic heart is more sensitive to ischemic injury. *Cardiovasc Res* 1997 **34**: 104-112.

Perseghin G, Scifo P, Pagliato E, Battezzati A, Benedini S, Soldini L, Testolin G, Del Maschio A, and Luzzi L. Gender factors affect fatty acids-induced insulin resistance in nonobese humans: Effects of oral steroidal contraception. *J Clin Endocrinol Metab* 2001 **86**: 3188-3196.

Pomerleau J, McKeigue PM, and Chaturvedi N. Relationships of fasting and postload glucose levels to sex and alcohol consumption. Are american diabetes association criteria biased against detection of diabetes in women? *Diabetes Care* 1999 **22**: 430-433.

Porrello ER, D'Amore A, Curl CL, Allen AM, Harrap SB, Thomas WG, and Delbridge LM. Angiotensin ii type 2 receptor antagonizes angiotensin ii type 1 receptor-mediated cardiomyocyte autophagy. *Hypertension* 2009 **53**: 1032-1040.

Rabi DM, Khan N, Vallee M, Hladunewich MA, Tobe SW, and Pilote L. Reporting on sex-based analysis in clinical trials of angiotensin-converting enzyme inhibitor and angiotensin receptor blocker efficacy. *Can J Cardiol* 2008 **24**: 491-496.

Radovits T, Korkmaz S, Loganathan S, Barnucz E, Bomicke T, Arif R, Karck M, and Szabo G. Comparative investigation of the left ventricular pressure-volume relationship in rat models of type 1 and type 2 diabetes mellitus. *Am J Physiol Heart Circ Physiol* 2009 **297**: H125-133.

Raimondi L, De Paoli P, Mannucci E, Lonardo G, Sartiani L, Banchelli G, Pirisino R, Mugelli A, and Cerbai E. Restoration of cardiomyocyte functional properties by angiotensin ii receptor blockade in diabetic rats. *Diabetes* 2004 **53**: 1927-1933.

Ravingerova T, Neckar J, and Kolar F. Ischemic tolerance of rat hearts in acute and chronic phases of experimental diabetes. *Mol Cell Biochem* 2003 **249**: 167-174.

Regan TJ, Lyons MM, Ahmed SS, Levinson GE, Oldewurtel HA, Ahmad MR, and Haider B. Evidence for cardiomyopathy in familial diabetes mellitus. *The Journal of clinical investigation* 1977 **60**: 884-899.

Reichelt M, Mellor K, Stapleton D, and Delbridge L. Glycogen responses during metabolic stress are accentuated in female hearts: A role for myocardial glycophagy. *Heart, Lung and Circulation* 2013a **22**: S63.

Reichelt ME, Mellor KM, Curl CL, Stapleton D, and Delbridge LM. Myocardial glycophagy - a specific glycogen handling response to metabolic stress is accentuated in the female heart. *J Mol Cell Cardiol* 2013b **65**: 67-75.

Ren J, Wold LE, and Epstein PN. Diabetes enhances acetaldehyde-induced depression of cardiac myocyte contraction. *Biochem Biophys Res Commun* 2000 **269**: 697-703.

Ren JY, Song JX, Lu MY, and Chen H. Cardioprotection by ischemic postconditioning is lost in isolated perfused heart from diabetic rats: Involvement of transient receptor potential vanilloid 1, calcitonin gene-related peptide and substance p. *Regul Pept* 2011 **169**: 49-57.

Ritchie RH, Love JE, Huynh K, Bernardo BC, Henstridge DC, Kiriazis H, Tham YK, Sapra G, Qin C, Cemerlang N, Boey EJ, Jandeleit-Dahm K, Du XJ, and McMullen JR. Enhanced phosphoinositide 3-kinase(p110alpha) activity prevents diabetes-induced cardiomyopathy and superoxide generation in a mouse model of diabetes. *Diabetologia* 2012 **55**: 3369-3381.

Ritchie RH, Quinn JM, Cao AH, Drummond GR, Kaye DM, Favalaro JM, Proietto J, and Delbridge LM. The antioxidant tempol inhibits cardiac hypertrophy in the insulin-resistant glut4-deficient mouse in vivo. *J Mol Cell Cardiol* 2007 **42**: 1119-1128.

Roach PJ, Depaoli-Roach AA, Hurley TD, and Tagliabracci VS. Glycogen and its metabolism: Some new developments and old themes. *Biochem J* 2012 **441**: 763-787.

Rodrigues B, and McNeill JH. Comparison of cardiac function in male and female diabetic rats. *Gen Pharmacol* 1987 **18**: 421-423.

Roe ND, Thomas DP, and Ren J. Inhibition of nadph oxidase alleviates experimental diabetes-induced myocardial contractile dysfunction. *Diabetes Obes Metab* 2011 **13**: 465-473.

Ruegg J, Cai W, Karimi M, Kiss NB, Swedenborg E, Larsson C, Ekstrom TJ, and Pongratz I. Epigenetic regulation of glucose transporter 4 by estrogen receptor beta. *Mol Endocrinol* 2011 **25**: 2017-2028.

Ryu JH, Drain J, Kim JH, McGee S, Gray-Weale A, Waddington L, Parker GJ, Hargreaves M, Yoo SH, and Stapleton D. Comparative structural analyses of purified glycogen particles from rat liver, human skeletal muscle and commercial preparations. *Int J Biol Macromol* 2009 **45**: 478-482.

Sakakibara M, Hirashiki A, Cheng XW, Bando Y, Ohshima K, Okumura T, Funahashi H, Ohshima S, and Murohara T. Association of diabetes mellitus with

myocardial collagen accumulation and relaxation impairment in patients with dilated cardiomyopathy. *Diabetes Res Clin Pract* 2011 **92**: 348-355.

Schaffer SW, Croft CB, and Solodushko V. Cardioprotective effect of chronic hyperglycemia: Effect on hypoxia-induced apoptosis and necrosis. *Am J Physiol Heart Circ Physiol* 2000 **278**: H1948-1954.

Schannwell CM, Schneppenheim M, Perings S, Plehn G, and Strauer BE. Left ventricular diastolic dysfunction as an early manifestation of diabetic cardiomyopathy. *Cardiology* 2002 **98**: 33-39.

Secrest AM, Becker DJ, Kelsey SF, Laporte RE, and Orchard TJ. Cause-specific mortality trends in a large population-based cohort with long-standing childhood-onset type 1 diabetes. *Diabetes* 2010 **59**: 3216-3222.

Seeman T, Mendes de Leon C, Berkman L, and Ostfeld A. Risk factors for coronary heart disease among older men and women: A prospective study of community-dwelling elderly. *Am J Epidemiol* 1993 **138**: 1037-1049.

Semeniuk LM, Kryski AJ, and Severson DL. Echocardiographic assessment of cardiac function in diabetic db/db and transgenic db/db-hglut4 mice. *Am J Physiol Heart Circ Physiol* 2002 **283**: H976-982.

Sernia C. A critical appraisal of the intrinsic pancreatic angiotensin-generating system. *J Pancreas* 2001 **2**: 50-55.

Shao CH, Capek HL, Patel KP, Wang M, Tang K, DeSouza C, Nagai R, Mayhan W, Periasamy M, and Bidasee KR. Carbonylation contributes to serca2a activity loss and diastolic dysfunction in a rat model of type 1 diabetes. *Diabetes* 2011 **60**: 947-959.

Shao CH, Rozanski GJ, Nagai R, Stockdale FE, Patel KP, Wang M, Singh J, Mayhan WG, and Bidasee KR. Carbonylation of myosin heavy chains in rat heart during diabetes. *Biochem Pharmacol* 2010 **80**: 205-217.

Sharma V, Sharma A, Saran V, Bernatchez PN, Allard MF, and McNeill JH. Beta-receptor antagonist treatment prevents activation of cell death signaling in the diabetic heart independent of its metabolic actions. *Eur J Pharmacol* 2011 **657**: 117-125.

Shearer J, Ross KD, Hughey CC, Johnsen VL, Hittel DS, and Severson DL. Exercise training does not correct abnormal cardiac glycogen accumulation in the db/db mouse model of type 2 diabetes. *Am J Physiol Endocrinol Metab* 2011 **301**: E31-39.

Shen JJ, and Chen YT. Molecular characterization of glycogen storage disease type iii. *Curr Mol Med* 2002 **2**: 167-175.

Shi-ting W, Mang-hua X, Wen-ting C, Feng-hou G, and Zhu-ying G. Study on tolerance to ischemia-reperfusion injury and protection of ischemic preconditioning of type 1 diabetes rat heart. *Biomed Pharmacol* 2010

Shimabukuro M, Higa S, Shinzato T, Nagamine F, Komiya I, and Takasu N. Cardioprotective effects of troglitazone in streptozotocin-induced diabetic rats. *Metabolism* 1996 **45**: 1168-1173.

Shimizu M, Umeda K, Sugihara N, Yoshio H, Ino H, Takeda R, Okada Y, and Nakanishi I. Collagen remodelling in myocardia of patients with diabetes. *Journal of clinical pathology* 1993 **46**: 32-36.

Shimoni Y, Chuang M, Abel ED, and Severson DL. Gender-dependent attenuation of cardiac potassium currents in type 2 diabetic db/db mice. *J Physiol* 2004 **555**: 345-354.

Shiomi T, Tsutsui H, Ikeuchi M, Matsusaka H, Hayashidani S, Suematsu N, Wen J, Kubota T, and Takeshita A. Streptozotocin-induced hyperglycemia exacerbates left ventricular remodeling and failure after experimental myocardial infarction. *J Am Coll Cardiol* 2003 **42**: 165-172.

Sicree RA, Zimmet PZ, Dunstan DW, Cameron AJ, Welborn TA, and Shaw JE. Differences in height explain gender differences in the response to the oral glucose tolerance test- the ausdiab study. *Diabet Med* 2008 **25**: 296-302.

Silva MF, Peluzio Mdo C, Amorim PR, Lavorato VN, Santos NP, Bozi LH, Penitente AR, Falkoski DL, Berfort FG, and Natali AJ. Swimming training attenuates contractile dysfunction in diabetic rat cardiomyocytes. *Arq Bras Cardiol* 2011 **97**: 33-39.

Simons LA, McCallum J, Friedlander Y, and Simons J. Diabetes, mortality and coronary heart disease in the prospective dubbo study of australian elderly. *Aust N Z J Med* 1996 **26**: 66-74.

Singh J, Chonkar A, Bracken N, Adeghate E, Latt Z, and Hussain M. Effect of streptozotocin-induced type 1 diabetes mellitus on contraction, calcium transient, and cation contents in the isolated rat heart. *Ann N Y Acad Sci* 2006 **1084**: 178-190.

Singh VP, Le B, Khode R, Baker KM, and Kumar R. Intracellular angiotensin ii production in diabetic rats is correlated with cardiomyocyte apoptosis, oxidative stress, and cardiac fibrosis. *Diabetes* 2008 **57**: 3297-3306.

Stapleton D, Nelson C, Parsawar K, McClain D, Gilbert-Wilson R, Barker E, Rudd B, Brown K, Hendrix W, O'Donnell P, and Parker G. Analysis of hepatic glycogen-associated proteins. *Proteomics* 2010 **10**: 2320-2329.

Strunz CM, Matsuda M, Salemi VM, Nogueira A, Mansur AP, Cestari IN, and Marquezini MV. Changes in cardiac heparan sulfate proteoglycan expression and streptozotocin-induced diastolic dysfunction in rats. *Cardiovasc Diabetol* 2011 **10**: 35.

Sun B, Fredrickson K, Austin S, Tolun AA, Thurberg BL, Kraus WE, Bali D, Chen YT, and Kishnani PS. Alglucosidase alfa enzyme replacement therapy as a therapeutic approach for glycogen storage disease type iii. *Mol Genet Metab* 2013 **108**: 145-147.

Suys BE, Katier N, Rooman RP, Matthys D, Op De Beeck L, Du Caju MV, and De Wolf D. Female children and adolescents with type 1 diabetes have more pronounced early echocardiographic signs of diabetic cardiomyopathy. *Diabetes Care* 2004 **27**: 1947-1953.

Tagliabracci VS, Girard JM, Segvich D, Meyer C, Turnbull J, Zhao X, Minassian BA, Depaoli-Roach AA, and Roach PJ. Abnormal metabolism of glycogen phosphate as a cause for lafora disease. *J Biol Chem* 2008 **283**: 33816-33825.

Tagliabracci VS, Heiss C, Karthik C, Contreras CJ, Glushka J, Ishihara M, Azadi P, Hurley TD, DePaoli-Roach AA, and Roach PJ. Phosphate incorporation during glycogen synthesis and lafora disease. *Cell Metab* 2011 **13**: 274-282.

Tagliabracci VS, Turnbull J, Wang W, Girard JM, Zhao X, Skurat AV, Delgado-Escueta AV, Minassian BA, Depaoli-Roach AA, and Roach PJ. Laforin is a glycogen phosphatase, deficiency of which leads to elevated phosphorylation of glycogen in vivo. *Proc Natl Acad Sci U S A* 2007 **104**: 19262-19266.

Takada JY, Ramos RB, Roza LC, Avakian SD, Ramires JA, and Mansur Ade P. In-hospital death in acute coronary syndrome was related to admission glucose in men but not in women. *Cardiovasc Diabetol* 2012 **11**: 47.

Takahashi N, Ooie T, Saikawa T, Iwao T, Yoshimatsu H, and Sakata T. Long-term treatment with glibenclamide increases susceptibility of streptozotocin-induced diabetic

rat heart to reperfusion-induced ventricular tachycardia. *Exp Biol Med (Maywood)* 2003 **228**: 1234-1238.

Tamis-Holland JE, Lu J, Bittner V, Magee MF, Lopes N, Adler DS, Kip KE, Schwartz L, Groenewoud YA, and Jacobs AK. Sex, clinical symptoms, and angiographic findings in patients with diabetes mellitus and coronary artery disease (from the bypass angioplasty revascularization investigation [bari] 2 diabetes trial). *Am J Cardiol* 2011 **107**: 980-985.

Tashiro K, Iwamasa T, Kato H, Ogata S, and Anai M. Purification and characterization of two components of acid alpha-glucosidase from pig liver. *J Biochem* 1986 **99**: 693-701.

Ti Y, Xie GL, Wang ZH, Bi XL, Ding WY, Wang J, Jiang GH, Bu PL, Zhang Y, Zhong M, and Zhang W. Trb3 gene silencing alleviates diabetic cardiomyopathy in a type 2 diabetic rat model. *Diabetes* 2011 **60**: 2963-2974.

Tian C, Shao CH, Moore CJ, Kutty S, Walseth T, DeSouza C, and Bidasee KR. Gain of function of cardiac ryanodine receptor in a rat model of type 1 diabetes. *Cardiovasc Res* 2011 **91**: 300-309.

Tosaki A, Engelman DT, Engelman RM, and Das DK. The evolution of diabetic response to ischemia/reperfusion and preconditioning in isolated working rat hearts. *Cardiovasc Res* 1996 **31**: 526-536.

Tsang A, Hausenloy DJ, Mocanu MM, Carr RD, and Yellon DM. Preconditioning the diabetic heart: The importance of akt phosphorylation. *Diabetes* 2005 **54**: 2360-2364.

Tuncay E, Seymen AA, Tanriverdi E, Yaras N, Tandogan B, Uluşu NN, and Turan B. Gender related differential effects of omega-3 treatment on diabetes-induced left ventricular dysfunction. *Mol Cell Biochem* 2007 **304**: 255-263.

Vaccarino V, Badimon L, Corti R, de Wit C, Dorobantu M, Hall A, Koller A, Marzilli M, Pries A, and Bugiardini R. Ischaemic heart disease in women: Are there sex differences in pathophysiology and risk factors? Position paper from the working group on coronary pathophysiology and microcirculation of the european society of cardiology. *Cardiovasc Res* 2011 **90**: 9-17.

Vadlamudi RV, Rodgers RL, and McNeill JH. The effect of chronic alloxan- and streptozotocin-induced diabetes on isolated rat heart performance. *Can J Physiol Pharmacol* 1982 **60**: 902-911.

van den Brom CE, Huisman MC, Vlasblom R, Boontje NM, Duijst S, Lubberink M, Molthoff CF, Lammertsma AA, van der Velden J, Boer C, Ouwens DM, and Diamant M. Altered myocardial substrate metabolism is associated with myocardial dysfunction in early diabetic cardiomyopathy in rats: Studies using positron emission tomography. *Cardiovasc Diabetol* 2009 **8**: 39.

van Heerebeek L, Hamdani N, Handoko ML, Falcao-Pires I, Musters RJ, Kupreishvili K, Ijsselmuiden AJ, Schalkwijk CG, Bronzwaer JG, Diamant M, Borbely A, van der Velden J, Stienen GJ, Laarman GJ, Niessen HW, and Paulus WJ. Diastolic stiffness of the failing diabetic heart: Importance of fibrosis, advanced glycation end products, and myocyte resting tension. *Circulation* 2008 **117**: 43-51.

van Heerebeek L, and Paulus WJ. Understanding heart failure with preserved ejection fraction: Where are we today? *Neth Heart J* 2016 **24**: 227-236.

VanHoose L, Sawers Y, Loganathan R, Vacek JL, Stehno-Bittel L, Novikova L, Al-Jarrah M, and Smirnova IV. Electrocardiographic changes with the onset of diabetes and the impact of aerobic exercise training in the Zucker diabetic fatty (ZDF) rat. *Cardiovasc Diabetol* 2010 **9**: 56.

Vasanji Z, Cantor EJ, Juric D, Moyan M, and Netticadan T. Alterations in cardiac contractile performance and sarcoplasmic reticulum function in sucrose-fed rats is associated with insulin resistance. *Am J Physiol Cell Physiol* 2006 **291**: C772-780.

Vecoli C, Cao J, Neglia D, Inoue K, Sodhi K, Vanella L, Gabrielson KK, Bedja D, Paolocci N, L'Abbate A, and Abraham NG. Apolipoprotein A-I mimetic peptide I-4f prevents myocardial and coronary dysfunction in diabetic mice. *J Cell Biochem* 2011 **112**: 2616-2626.

Velloso LA, Folli F, Perego L, and Saad MJ. The multi-faceted cross-talk between the insulin and angiotensin II signaling systems. *Diabetes Metab Res Rev* 2006 **22**: 98-107.

Venskutonyte L, Malmberg K, Norhammar A, Wedel H, and Ryden L. Effect of gender on prognosis in patients with myocardial infarction and type 2 diabetes. *J Intern Med* 2010 **268**: 75-82.

Vinson GP, Saridogan E, Puddefoot JR, and Djahanbakhch O. Tissue renin-angiotensin systems and reproduction. *Hum Reprod* 1997 **12**: 651-662.

- Vital P, Larrieta E, and Hiriart M.** Sexual dimorphism in insulin sensitivity and susceptibility to develop diabetes in rats. *J Endocrinol* 2006 **190**: 425-432.
- Wang P, and Chatham JC.** Onset of diabetes in Zucker diabetic fatty (ZDF) rats leads to improved recovery of function after ischemia in the isolated perfused heart. *Am J Physiol Endocrinol Metab* 2004 **286**: E725-736.
- Wang P, Lloyd SG, Zeng H, Bonen A, and Chatham JC.** Impact of altered substrate utilization on cardiac function in isolated hearts from Zucker diabetic fatty rats. *Am J Physiol Heart Circ Physiol* 2005 **288**: H2102-2110.
- Warren S.** The effect of insulin on pathologic glycogen deposits in diabetes mellitus. *Am J Med Sci* 1930 **179** pp. 482-488.
- Wei Z, Wang L, Han J, Song J, Yao L, Shao L, Sun Z, and Zheng L.** Decreased expression of transient receptor potential vanilloid 1 impairs the postischemic recovery of diabetic mouse hearts. *Circ J* 2009 **73**: 1127-1132.
- Westermann D, Rutschow S, Jager S, Linderer A, Anker S, Riad A, Unger T, Schultheiss HP, Pauschinger M, and Tschope C.** Contributions of inflammation and cardiac matrix metalloproteinase activity to cardiac failure in diabetic cardiomyopathy: The role of angiotensin type 1 receptor antagonism. *Diabetes* 2007 **56**: 641-646.
- Whittington HJ, Babu GG, Mocanu MM, Yellon DM, and Hausenloy DJ.** The diabetic heart: Too sweet for its own good? *Cardiol Res Pract* 2012 **2012**: 845698.
- Widom B, Diamond MP, and Simonson DC.** Alterations in glucose metabolism during menstrual cycle in women with IDDM. *Diabetes Care* 1992 **15**: 213-220.
- Williams JW, Zimmet PZ, Shaw JE, de Courten MP, Cameron AJ, Chitson P, Tuomilehto J, and Alberti KG.** Gender differences in the prevalence of impaired fasting glycaemia and impaired glucose tolerance in Mauritius. Does sex matter? *Diabet Med* 2003 **20**: 915-920.
- Wold LE, Ceylan-Isik AF, Fang CX, Yang X, Li SY, Sreejayan N, Privratsky JR, and Ren J.** Metallothionein alleviates cardiac dysfunction in streptozotocin-induced diabetes: Role of Ca²⁺ cycling proteins, NADPH oxidase, poly(ADP-ribose) polymerase and myosin heavy chain isozyme. *Free radical biology & medicine* 2006 **40**: 1419-1429.

Wold LE, Relling DP, Colligan PB, Scott GI, Hintz KK, Ren BH, Epstein PN, and Ren J. Characterization of contractile function in diabetic hypertensive cardiomyopathy in adult rat ventricular myocytes. *J Mol Cell Cardiol* 2001 **33**: 1719-1726.

Xiao CY, Chen M, Zsengeller Z, and Szabo C. Poly(adp-ribose) polymerase contributes to the development of myocardial infarction in diabetic rats and regulates the nuclear translocation of apoptosis-inducing factor. *J Pharmacol Exp Ther* 2004 **310**: 498-504.

Xie Z, Lau K, Eby B, Lozano P, He C, Pennington B, Li H, Rath S, Dong Y, Tian R, Kem D, and Zou MH. Improvement of cardiac functions by chronic metformin treatment is associated with enhanced cardiac autophagy in diabetic ove26 mice. *Diabetes* 2011 **60**: 1770-1778.

Xu F, Ding E, Migone F, Serra D, Schneider A, Chen YT, and Amalfitano A. Glycogen storage in multiple muscles of old gsd-ii mice can be rapidly cleared after a single intravenous injection with a modified adenoviral vector expressing hgaa. *J Gene Med* 2005 **7**: 171-178.

Yamada T, Akishita M, Pollman MJ, Gibbons GH, Dzau VJ, and Horiuchi M. Angiotensin ii type 2 receptor mediates vascular smooth muscle cell apoptosis and antagonizes angiotensin ii type 1 receptor action: An in vitro gene transfer study. *Life Sci* 1998 **63**: P1289-295.

Yang SW, Zhou YJ, Nie XM, Liu YY, Du J, Hu DY, Jia DA, Gao F, Hu B, Fang Z, Han HY, Liu XL, Yan ZX, Wang JL, Hua Q, Shi YJ, and Li HW. Effect of abnormal fasting plasma glucose level on all-cause mortality in older patients with acute myocardial infarction: Results from the beijing elderly acute myocardial infarction study (beamis). *Mayo Clin Proc* 2011 **86**: 94-104.

Yang Z, and Vatta M. Danon disease as a cause of autophagic vacuolar myopathy. *Congenit Heart Dis* 2007 **2**: 404-409.

Yaras N, Tuncay E, Purali N, Sahinoglu B, Vassort G, and Turan B. Sex-related effects on diabetes-induced alterations in calcium release in the rat heart. *Am J Physiol Heart Circ Physiol* 2007 **293**: H3584-3592.

Yu X, Tesiram YA, Towner RA, Abbott A, Patterson E, Huang S, Garrett MW, Chandrasekaran S, Matsuzaki S, Szweda LI, Gordon BE, and Kem DC. Early myocardial dysfunction in streptozotocin-induced diabetic mice: A study using in vivo magnetic resonance imaging (mri). *Cardiovasc Diabetol* 2007 **6**: 6.

Yusuf S, Gerstein H, Hoogwerf B, Pogue J, Bosch J, Wolffenbuttel BH, and Zinman B. Ramipril and the development of diabetes. *Jama* 2001 **286**: 1882-1885.

Zabaloitia M, Ismaeil MF, Anderson L, and Maklady FA. Prevalence of diastolic dysfunction in normotensive, asymptomatic patients with well-controlled type 2 diabetes mellitus. *Am J Cardiol* 2001 **87**: 320-323.

Zhang J, Zhang BH, Yu YR, Tang CS, and Qi YF. Adrenomedullin protects against fructose-induced insulin resistance and myocardial hypertrophy in rats. *Peptides* 2011a **32**: 1415-1421.

Zhang X, Dostal DE, Reiss K, Cheng W, Kajstura J, Li P, Huang H, Sonnenblick EH, Meggs LG, Baker KM, and et al. Identification and activation of autocrine renin-angiotensin system in adult ventricular myocytes. *Am J Physiol* 1995 **269**: H1791-1802.

Zhang YF, Xiao K, Chandramouli KH, Xu Y, Pan K, Wang WX, and Qian PY. Acute toxicity of the antifouling compound butenolide in non-target organisms. *PLoS One* 2011b **6**: e23803.

Zhou H, Li YJ, Wang M, Zhang LH, Guo BY, Zhao ZS, Meng FL, Deng YG, and Wang RY. Involvement of rhoa/rock in myocardial fibrosis in a rat model of type 2 diabetes. *Acta Pharmacol Sin* 2011 **32**: 999-1008.

Zhu Y, Jiang JL, Gumlaw NK, Zhang J, Bercury SD, Ziegler RJ, Lee K, Kudo M, Canfield WM, Edmunds T, Jiang C, Mattaliano RJ, and Cheng SH. Glycoengineered acid alpha-glucosidase with improved efficacy at correcting the metabolic aberrations and motor function deficits in a mouse model of pompe disease. *Mol Ther* 2009 **17**: 954-963.

Zhu Y, Zhang M, Kelly AR, and Cheng A. The carbohydrate-binding domain of overexpressed stbd1 is important for its stability and protein-protein interactions. *Biosci Rep* 2014

Zibadi S, Cordova F, Slack EH, Watson RR, and Larson DF. Leptin's regulation of obesity-induced cardiac extracellular matrix remodeling. *Cardiovasc Toxicol* 2011 **11**: 325-333.

CHAPTER 2

General Methodology

2.1 Experimental animals

All animals used in this study were handled in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (NHMRC/CSIRO/ACC, 2013). All the procedures carried out as part of this study were approved by the Animal Ethics Committee of The University of Melbourne (AEC Approval Numbers: 1112184, 1101838, 1101784). Animals were housed in the Biomedical Science Animal Facility at the University of Melbourne in polypropylene cages in a temperature controlled facility on a 12:12 hour light and dark cycle. Mice were provided access to water and standard chow *ad libitum*.

2.2 Induction of acute insulin deficiency by Streptozotocin in mice

To examine the effects of the early onset of insulin deficiency, acute insulin withdrawal (Acute STZ) was induced in 8 week-old male and female C57Bl/6 mice. Acute T1D was induced via 5 daily intraperitoneal injections of 55 mg/kg Streptozotocin (STZ; Sigma-Aldrich, MO, USA). STZ stock (5.5 mg/mL) was prepared fresh everyday in 0.1M tri-sodium citrate buffer (pH 4.5, 4°C) (Sigma-Aldrich, MO, USA). Vehicle control mice received 5 tri-sodium citrate buffer injections. Body weight and blood glucose was measured prior to injections (day 0) and on days 1, 3, 5, 7 and 9 by glucometer (ACCU-CHEK® Advantage glucometer, Roche, Mannheim, Germany). Body weight, food and water intake was monitored daily throughout injection period and for ~1 week (depending on time taken to reach 50% increase in blood glucose) after STZ administration. Cage bedding of STZ mice was changed daily throughout the injection period. All animal care and STZ usage standards specified by the Office of Research Ethics and Integrity at the University of Melbourne were adhered to throughout the experimental period. Following blood glucose levels reaching 50% increase from baseline (2 days in males and 7 days in female mice post STZ injections), mice were culled and samples were collected as described in Section 2.8. Findings are detailed in Chapter 3 Results Section 2.2B and discussed in Chapter 3 Discussion Section 3.4A.

2.3 The Angiotensinogen-Transgenic (AngII-Tg) mouse

The cardiac-specific angiotensinogen overexpressing mouse strain (Tg1306/IR) used in this study was generated by Pedrazzini and colleagues (Laussane, Switzerland) through

insertion of 30 copies of the rat angiotensinogen gene into the mouse genome using the cardiac promoter, α -myosin heavy chain (α -MHC) (Mazzolai *et al.*, 1998; Mazzolai *et al.*, 2000). Heterozygous males (Tg1306/IR) were bred with wildtype C57Bl/6 female breeders to generate litters of heterozygous transgenic mice with elevated cardiac angiotensin-II levels (AngII-Tg) and wildtype littermate controls.

The AngII-Tg model is characterised by cardiac hypertrophy with an elevated cardiac weight index (ratio of ventricular wet weight (mg) to body weight (g)) by 8 weeks of age (Mazzolai *et al.*, 1998), and alterations in cardiomyocyte contractile properties (Domenighetti *et al.*, 2007; Huggins *et al.*, 2003), in the absence of increased fibrosis or hypertension (Mazzolai *et al.*, 1998; Mazzolai *et al.*, 2000). The AngII-Tg mouse exhibits increased mortality (83% in Wt vs. 46% in AngII-Tg), but not until the age of 45 weeks (Domenighetti *et al.*, 2005). Therefore, this model allows examination of cardiac growth resulting from elevated myocardial AngII independent of any influence from hemodynamic loading.

2.4 Induction of chronic insulin deficiency by Streptozotocin in mice

To examine the pathophysiology of chronic T1D, STZ was administered (as described in Section 2.2) to Wildtype and AngII-Tg mice of both sexes at 15 weeks of age and diabetes development was investigated for subsequent 8 weeks. Body weight and food intake were monitored throughout the 8 weeks diabetic period and blood glucose was measured before STZ injections and at 7 weeks post STZ injections. All functional experiments described in subsequent sections were performed in these chronic T1D mice.

2.5 Glucose Tolerance Testing

At 8 weeks post STZ injections, glucose tolerance testing was performed on chronic T1D mice following 6 hours fasting as described previously (Mellor *et al.*, 2011). A tail prick (23G needle) was used to collect blood samples (~5 μ L) for measurement using a glucometer (ACCU-CHEK® Advantage glucometer, Roche, Mannheim, Germany). Following baseline blood glucose measurement, 1.5 mg/g glucose solution was injected (27G needle, i.p.) and blood glucose was measured at 5, 15, 30, 60, 90 and 120 mins post-injection. Area under the curve was subsequently calculated using trapezoidal integration as an index of glucose disposal from plasma (Sakaguchi *et al.*, 2016).

2.6 Echocardiography assessment of *in vivo* cardiac function

Transthoracic echocardiography was undertaken, as described previously (Reichelt *et al.*, 2013), in mice at 7 weeks post STZ injections under light anaesthesia with tribromethanol (Dosage: 2.5%, 0.01ml/g i.p). The GE Vivid 9 Dimension echocardiography system with a 15 MHz i13L linear array transducer (GE Healthcare, CT, USA) was used. Left ventricular *m-mode* two-dimensional echocardiography was performed in parasternal short axis view to measure left ventricular chamber dimensions, wall thickness and to derive systolic function. In addition, Pulse wave (PW) Doppler and Tissue Doppler Imaging (TDI) were acquired from the apical 4 chamber views to measure transmitral flow propagation velocity inflow using loading-dependent and independent measures respectively.

Acquisition and offline analysis was performed using the GE EchoPac software. M-mode derived echocardiography parameters included diastolic and systolic interventricular septal dimensions (IVSd, IVSs), diastolic and systolic left ventricular dimensions (LVIDd, LVIDs) and diastolic and systolic left ventricular posterior wall thickness (LVPWd, LVPWs). Three consecutive echo-loops were sampled for each measurement taken. Pulse wave Doppler images were used to measure the velocity of early mitral inflow (E) and late mitral inflow (atrial inflow A). The ratio of E/A, an indicator of diastolic function, was subsequently calculated. TVI Doppler images were used to measure mitral valve annulus movement (loading-independent tissue velocity). These include early diastolic velocity of mitral annulus (E') and late diastolic velocity of mitral annulus (atrial contraction velocity A'). The ratio of E/E' and E'/A' are also used to estimate diastolic dysfunction and LV end diastolic pressure.

2.7 *Ex vivo* cardiac function and ischemia-reperfusion

At 8 weeks post-diabetes induction, Wildtype and AngII-Tg mice were subjected to *ex vivo* ischemia reperfusion as described previously (Headrick *et al.*, 2001; Reichelt *et al.*, 2007). Briefly, mice were anesthetised through intraperitoneal administration of sodium pentobarbitone (70 mg/kg, i.p). A thoracotomy was performed and hearts were rapidly excised into ice-cold perfusate solution. The aorta was cannulated and hearts were retrogradely perfused with oxygenated (95% O₂, 5% CO₂) modified Krebs-Henselitt buffer (37.0°C, pH 7.4) in Langendorff mode at a constant pressure of 80 mmHg and instrumented for pressure recording (ADInstruments, Labchart v7.2). The modified

Krebs-Henselit buffer contained (in mmol per litre): 119.0 NaCl, 11.0 glucose, 22.0 NaHCO₃, 4.7 KCL, 1.2 MgCl, 1.2 KH₂PO₄, 0.5 EDTA, 1.75 CaCl, 2.0 Na pyruvate. Following a 15 mins stabilisation period, pacing was commenced at 420 beats per minute and stabilised for a further 15 mins. Hearts were excluded from analysis at this time if they met one of the exclusion criteria listed below:

- I. left ventricular systolic pressure <100 mmHg
- II. coronary flow $\geq 5 \text{ ml min}^{-1}$
- III. fluctuating contractile function
- IV. significant arrhythmias

Baseline functional measurements were made before the hearts were subjected to 20 mins of global no flow ischemia. Pacing was stopped upon induction of ischemia. Subsequently, hearts were reperfused for 45 mins and pacing was resumed at 2 mins of reperfusion. Arrhythmia incidence was assessed from left ventricle pressure recordings as previously validated from electrocardiogram measurements. Hearts were removed from the apparatus. A transverse section ($\sim 5 \mu\text{m}$) was cut at the mid point of the heart apex-to-base aspect ratio and fixed in formalin overnight for histological analysis, the remainder was snap frozen for protein and mRNA analysis.

2.8 Tissue, blood and plasma collection

Upon completion of STZ treatment, mice were anesthetised using sodium pentobarbitone (100 mg/kg i.p.). Prior to heart excision, heparin (100 I.U., i.p.) was injected (22G needle) into the inferior vena cava and a blood sample ($\sim 400 \mu\text{L}$) was drawn into a 1 mL syringe. A portion of the blood collected (100 μL) was stored at 4°C for HbA1c analysis and 300 μL was centrifuged (2000 g; 20 mins) to obtain plasma ($\sim 100 \mu\text{L}$) which was stored at -80°C until further analysis. Hearts were rapidly excised and arrested in iced HEPES-Krebs buffer (146 mM NaCl, 4.7 mM KCl, 1 mM Glucose, 0.3 mM NaH₂PO₄, 1 mM MgSO₄, 10 mM HEPES; pH 7.55; 4°C). Ventricular tissues were blotted on filter paper, weighed on an analytical balance and snap frozen in liquid nitrogen to be stored at -80°C until further analysis. Tibia lengths were measured using electronic digital calipers.

2.9 Insulin ELISA measurement

Plasma insulin levels were measured using an ultrasensitive mouse insulin ELISA method (Crystal Chem, IL, USA) (Chhabra *et al.*, 2016; Davidson *et al.*, 2016).

Samples were incubated in an antibody-coated microplate for 2 hours at 4°C to allow optimal binding. Wells were washed five times with 300 µL wash buffer and incubated with anti-insulin enzyme conjugate for 30 mins at room temperature. Wells were then washed seven times with 300 µL wash buffer and incubated with 100 µL enzyme substrate solution at room temperature for 10 mins while avoiding light exposure. Absorbance was measured at A₄₅₀ and A₆₃₀ nm using a microplate spectrophotometer (Biorad, CA, USA). Individual sample and standard absorbance was determined by subtracting A₄₅₀-A₆₃₀. Plasma insulin concentrations (ng/mL) were determined in reference to mouse insulin standards provided in the kit.

2.10 Glycated haemoglobin measurement

Haemoglobin A1c (HbA1c) was measured using cobas b101 with a cartridge-based point-of-care system (Roche, Basel, Switzerland) at the Baker IDI Heart and Diabetes Institute in collaboration with Associate Professor Rebecca Ritchie and Dr. Miles De Blasio from the Heart Failure Pharmacology Laboratory. Briefly, a small volume of heparinised blood (2 µL) was mixed with TRIS buffer within the cobas HbA1c Test cartridge (Roche, Basel, Switzerland) to release haemoglobin from erythrocytes. Introduction of a sodium lauryl sulphate (SLS) buffer within the cartridge led to formation of SLS-Hb complex and total haemoglobin (Hb) was detected by measuring the concentration of the SLS-Hb complex at 525 nm. A separate portion of the sample was used to measure the concentration of HbA1c fraction in another section of the cartridge by means of latex agglutination immunoturbimetric reaction at 625 nm. The percentage HbA1c value was calculated using a ratio of concentrations of HbA1c to total haemoglobin.

2.11 Protein extraction through tissue homogenisation

Frozen ventricular tissues were homogenised in buffer (Tris-HCL 100 mM pH 7.0, EGTA 5 mM; Sigma-Aldrich, MO, USA) (4°C, 5% w/v) containing phosphatase and proteinase inhibitors (Roche, Basel, Switzerland) in three bursts of 15secs/each using a Polytron PT2500E with a PT-DA 1207/2EC-E Dispersing aggregate (Kinematica, Luzern, Switzerland). Homogenate was snap frozen in liquid N₂ immediately for glycogen content and protein concentration determination.

2.12 Protein concentration determination

Protein concentrations in tissue homogenate were determined using the standard Lowry assay (Peterson 1977). Diluted (1:1000) crude homogenates were incubated in Reagent A (10% SDS, 0.8M NaOH, 12.5% Na₂CO₃, 2% Na₂CO₃, 2% Na⁺ K⁺ Tartarate, 1%CuSO₄) at room temperature for 10 mins. Subsequently, samples were incubated in Folin-Ciocalteu phenol reagent (Reagent B, Biorad, CA, USA) at room temperature for 30 mins resulting in a colour change from yellow to blue. Absorbance was measured at 750 nm and protein concentrations were determined with reference to the standard curve generated with bovine serum albumin (Biorad, CA, USA).

2.13 Enzymatic analysis of glycogen content

Cardiac glycogen concentrations were measured as described previously (Reichelt *et al.*, 2013). Crude tissue homogenates were incubated in the presence or absence of amyloglucosidase at 50°C for 60 mins in a buffer containing 0.1 M sodium acetate pH 6.0 and 1% Triton. Enzyme-digested and undigested samples were centrifuged at 14,000g for 2 mins, the supernatant was added to glucose oxidase/peroxidase (PGO) enzyme (Sigma-Aldrich, MO, USA) and *o*-dianisidine dihydrochloride (Sigma-Aldrich, MO, USA) solution. Following incubation at room temperature for 30 mins while avoiding exposure to light, the intensity of colour change, which is proportional to glucose concentration, was measured at 450 nm. Glucose concentration from the undigested sample was subtracted from the amyloglucosidase-digested sample to derive glycogen concentrations (nM) with reference to the standard curve generated from a glucose standard solution (Sigma-Aldrich, MO, USA).

2.14 RNA extraction and quantification

RNA was extracted from snap-frozen ventricular tissues using the TRIzol® reagent method followed by purification using the PureLink RNA mini kit (Invitrogen, CA, USA) (Mellor *et al.*, 2014). Briefly, tissues (50-100 mg) were homogenised in 1 mL of TRIzol® (Invitrogen, CA, USA) and incubated at room temperature for 5 mins to allow dissociation of the nucleoprotein complex. Chloroform (0.2 mL) was added and samples were shaken vigorously using a foam tube holder. Following incubation at room temperature for 2-3 mins, samples were centrifuged at 12000 g for 15 mins at 4°C. The aqueous phase supernatant was added to equal volumes of 70% ethanol. The extracted

RNA was purified as per the manufacturer's instructions (PureLink® RNA Mini Kit, Invitrogen CA, USA) and DNase-treated on column (*PureLink® DNase*, Invitrogen CA, USA). Purified RNA was eluted in 30 µL of nuclease-free water. RNA purity and yield were analysed using spectrometry (Nanodrop, Thermo Fisher Scientific, MA, USA). A 260/280 nm ratio of 1.90-2.10 was accepted as pure RNA with least contamination of proteins and phenols as the latter absorb strongly at 280 nm. In addition, the 260/230 nm ratio was also used to determine RNA quality, where values between 2.0-2.2 were considered acceptable. Remnants of carbohydrates, EDTA and phenols, which absorb at 230 nm, would result in a ratio of <2.0 indicating contamination in RNA samples. RNA samples that fell outside these acceptable ranges were excluded from further analysis. Ribosomal RNA band integrity was verified by running an aliquot of RNA sample on a 2% denaturing agarose gel. Identification of sharp bands/peaks of 18S and 28S ribosomal RNA indicated that the RNA was intact. RNA quality was also assessed using the RT² RNA Quality Control PCR Array plate (Qiagen, CA, USA) before gene expression analysis using RT² PCR Array (Qiagen, CA, USA) was performed (Appendix 2). Substandard RNA samples were excluded prior to gene expression analysis with RT² Profiler PCR Arrays (See Section 3.5).

2.15 Reverse transcription

Reverse transcription of purified RNA into cDNA was performed using the RT² First Strand Kit (Qiagen, CA, USA) using instructions provided by the manufacturer. Briefly, RNA (1 µg) was incubated at 42°C with a gDNA elimination buffer for 5 mins to eliminate genomic DNA contamination. Samples were immediately cooled at 4°C for 1 min and subsequently incubated in reverse transcriptase mix (4µL 5XBuffer BC3, 1µL Control P2, 2µL RE3 Reverse Transcriptase Mix, 3µL RNase-free water) at 42°C for 15 mins. The reaction was stopped by incubating samples at 95°C for 5 mins. RNase-free water (91µL) was added to each tube and cDNA was stored at the -20°C freezer until required.

2.16 RT profiling gene expression analysis

Gene expression analysis was evaluated using a customised mouse RT² PCR Array (Qiagen, CA, USA) on a Biorad CFX384 (Biorad, CA, USA) thermocycler. RT² Profiler PCR Arrays take advantage of the combination of real-time PCR performance

and the ability of microarrays to detect the expression of many genes simultaneously (Munasinghe *et al.*, 2016). The RT² 384 PCR Array was customised with pre-defined primer assays in a 64 wells x 6 replicate formats (Qiagen Cat#: CAPM12962). These include 59 genes of interest (refer Table 2.1), 2 reference genes (18S and ACTB), a genomic DNA control, a reverse transcription control (RTC) and a positive PCR control (PPC). Real-time PCR component mixes were prepared in 6 sets for 6 samples (e.g. n=3 Ctrl and n=3 STZ) on the RT² plate. PCR mastermix contained 353µL of 2x RT² SYBR Green Mastermix (Qiagen, CA, USA), 55.2µL cDNA and 296.8µL of nuclease-free water (Ambion, Texas, USA). 10 µL of the PCR component mix (containing the sample) was carefully dispensed into each well and mixed thoroughly with the pre-plated primers. Following plating of 384 wells, the RT² Profiler plate was sealed tightly with Optical Adhesive Film (Qiagen, CA, USA). The RT² Profiler plate was centrifuged for 1 min at 1000 g at room temperature to ensure the solution was at the bottom of the well and subsequently placed in the Biorad CFX384 real-time thermocycler (Biorad, CA, USA). Thermal cycling conditions for real-time PCR were 1 min at 95°C, 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C each, with a ramp rate of 1°C/s.

Data exported from the thermocycler were analysed using the Biorad CFX Manager Software (Version 3.1) (Biorad, CA, USA). Threshold was defined manually using the log scale of the amplification plots displayed. This threshold value was kept consistent between all RT² Profiler PCR Array runs performed. Ct values were exported and analysed using the comparative $\Delta\Delta\text{Ct}$ method (Livak *et al.*, 2001). Expression of the gene of interest was normalised to either 18S or β -actin (ACTB) housekeeper expression. The ΔCt for each sample was then further normalised to the ΔCt of a control sample from the same treatment group.

$$\Delta\text{Ct} = \text{Ct (gene of interest)} - \text{Ct (housekeeping gene)}$$

$$\Delta\Delta\text{Ct} = \Delta\text{Ct (test sample)} - \Delta\text{Ct (control sample)}$$

The last step in quantification was to transform the $\Delta\Delta\text{Ct}$ values to absolute values. The formula used for the comparative expression level was $2^{-\Delta\Delta\text{Ct}}$, which allows gene expression levels to be presented as a fold-change relative to the calibrator sample.

Table 2.1. List of genes included in the RT² Profiler PCR Array plate (n=64 genes).

Refseq #	Gene Symbol	Official Full Name	Catalogue Number
NM_020590	Gabarapl1	Gamma-aminobutyric acid -A receptor-associated protein-like 1	PPM25802
NM_175096	Stbd1	Starch binding domain 1	PPM26240
NM_019584	Becn1	Beclin 1, autophagy related	PPM32434
NM_008064	Gaa	Glucosidase, alpha, acid	PPM25180
NM_026160	Map1lc3b	Microtubule-associated protein 1 light chain 3 beta	PPM27261
NM_020009	Mtor	Mechanistic target of rapamycin (serine/threonine kinase)	PPM05092
NM_001013367	Prkaa1	Protein kinase, AMP-activated, alpha 1 catalytic subunit	PPM33117
NM_011018	Sqstm1	Sequestosome 1	PPM28731
NM_009469	Ulk1	Unc-51 like kinase 1 (C. elegans)	PPM36453
NM_010685	Lamp2	Lysosomal-associated membrane protein 2	PPM24540
NM_001081326	Agl	Amylo-1,6-glucosidase, 4-alpha-glucanotransferase	PPM34374
NM_001031667	Gsk3a	Glycogen synthase kinase 3 alpha	PPM05437
NM_019827	Gsk3b	Glycogen synthase kinase 3 beta	PPM03380
NM_030678	Gys1	Glycogen synthase 1, muscle	PPM03506
NM_145572	Gys2	Glycogen synthase 2	PPM36932
NM_173021	Phka1	Phosphorylase kinase alpha 1	PPM33414
NM_199446	Phkb	Phosphorylase kinase beta	PPM34377
NM_011079	Phkg1	Phosphorylase kinase gamma 1	PPM24927
NM_011224	Pygm	Muscle glycogen phosphorylase	PPM27122
NM_021514	Pfkm	Phosphofructokinase, muscle	PPM36557
NM_029846	Atg16l1	Autophagy-related 16-like 1 (yeast)	PPM36606
NM_001111111	Atg16l2	Autophagy related 16 like 2 (S. cerevisiae)	PPM36891
NM_026402	Atg3	Autophagy-related 3 (yeast)	PPM28898
NM_174874	Atg4b	Autophagy-related 4B (yeast)	PPM27447
NM_175029	Atg4c	Autophagy-related 4C (yeast)	PPM37063
NM_053069	Atg5	Autophagy-related 5 (yeast)	PPM26274
NM_028835	Atg7	Autophagy-related 7 (yeast)	PPM36835
NM_001003917	Atg9a	Autophagy-related 9A (yeast)	PPM58840
NM_007522	Bad	BCL2-associated agonist of cell death	PPM02916
NM_009741	Bcl2	B-cell leukemia/lymphoma 2	PPM02918
NM_009743	Bcl2l1	Bcl2-like 1	PPM02920
NM_009760	Bnip3	BCL2/adenovirus E1B interacting protein 3	PPM03409
NM_009810	Casp3	Caspase 3	PPM02922
NM_007798	Ctsb	Cathepsin B	PPM03626
NM_009983	Ctsd	Cathepsin D	PPM03622
NM_021281	Ctss	Cathepsin S	PPM05788
NM_029653	Dapk1	Death associated protein kinase 1	PPM26547
NM_026013	Dram2	VDNA-damage regulated autophagy modulator 2	PPM37764
NM_019749	Gabarap	Gamma-aminobutyric acid receptor-associated protein	PPM36541
NM_026693	Gabarapl2	Gamma-aminobutyric acid -A receptor-associated protein-like 2	PPM35460
NM_010512	Igf1	Insulin-like growth factor 1	PPM03387
NM_008387	Ins2	Insulin II	PPM25215
NM_010684	Lamp1	Lysosomal-associated membrane protein 1	PPM25844
NM_025735	Map1lc3a	Microtubule-associated protein 1 light chain 3 alpha	PPM32828
NM_181414	Pik3c3	Phosphoinositide-3-kinase, class 3	PPM32760
NM_020272	Pik3cg	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	PPM03469
NM_001081309	Pik3r4	Phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 4, p150	PPM05099
NM_026880	Pink1	PTEN induced putative kinase 1	PPM25933
NM_145940	Wip1	WD repeat domain, phosphoinositide interacting 1	PPM28369
NM_010292	Gck	Glucokinase	PPM05095
NM_008155	Gpi1	Glucose phosphate isomerase 1	PPM03345
NM_013820	Hk2	Hexokinase 2	PPM03503
NM_025700	Pgm1	Phosphoglucomutase 1	PPM24785
NM_028132	Pgm2	Phosphoglucomutase 2	PPM39901
NM_028352	Pgm3	Phosphoglucomutase 3	PPM26707
NM_139297	Ugp2	UDP-glucose pyrophosphorylase 2	PPM27391
NM_009652	Akt1	Thymoma viral proto-oncogene 1	PPM03377
NM_172669	Ambra1	Autophagy/beclin 1 regulator 1	PPM25885
NM_026217	Atg12	Autophagy-related 12 (yeast)	PPM25545
NM_007393	Actb	Actin, beta	PPM02945
NR_003278	Rn18s	18S ribosomal RNA	PPM72041
SA_00106	MGDC	Mouse Genomic DNA Contamination	PPM65836
SA_00104	RTC	Reverse Transcription Control	PPX63340
SA_00103	PPC	Positive PCR Control	PPX63339

2.17 Picrosirius Red staining

A transverse cross section of ventricular myocardium (~2 mm) was fixed in 10% formalin. Specimens were processed for paraffin embedding and sectioned in 6 µm-thickness. Slides were stained using Picrosirius Red (Picric Acid (Sigma-Aldrich, MO, USA), 0.1% Sirius Red (BDH AnalaR, England)) for 60 mins, dehydrated and mounted with DPX medium (Sigma-Aldrich, MO, USA). Brightfield microscopic images were captured with using the Zeiss Imager D1 microscope connected to a Zeiss AxioCam MRc5 Colour Camera (Oberkochen, Germany) and AxioVision 40 acquisition software (version 4.7.1.0) with 10 images per section, from 3 sections per heart. Image analysis was performed in a blinded manner using Image Pro Plus (V4.5.1, Media Cybernetics, MD, USA). Images underwent grey scale (255 pixel range) transformation and a pixel intensity histogram was assessed to determine the non-bias threshold point of collagen staining. A binary map of collagen deposition was generated, from which collagen density (shown in “red”) was calculated and expressed relative to the total number of pixels in the area of interest (Mellor *et al.*, 2011).

2.18 Statistical analysis

Data is presented as mean ± sem. Statistical analyses were performed using GraphPad Prism V7.0 (GraphPad, CA, USA). Normal distribution of data was assessed using Shapiro-Wilk’s test and homogeneity of variance was ensured using Levene’s test. Data comparisons between more than 2 groups with 2 factors were conducted by two-way ANOVA and Fisher’s LSD post-hoc test. Comparisons between two groups were analysed using unpaired Student t-tests where appropriate. A p-value of <0.05 was considered statistically significant.

2.19 References

Chhabra KH, Adams JM, Fagel B, Lam DD, Qi N, Rubinstein M, and Low MJ. Hypothalamic pomc deficiency improves glucose tolerance despite insulin resistance by increasing glycosuria. *Diabetes* 2016 **65**: 660-672.

Davidson SM, Papagiannakopoulos T, Olenchock BA, Heyman JE, Keibler MA, Luengo A, Bauer MR, Jha AK, O'Brien JP, Pierce KA, Gui DY, Sullivan LB, Wasylenko TM, Subbaraj L, Chin CR, Stephanopolous G, Mott BT, Jacks T, Clish CB, and Vander Heiden MG. Environment impacts the metabolic dependencies of ras-driven non-small cell lung cancer. *Cell Metab* 2016 **23**: 517-528.

Domenighetti AA, Boixel C, Cefai D, Abriel H, and Pedrazzini T. Chronic angiotensin ii stimulation in the heart produces an acquired long qt syndrome associated with ik1 potassium current downregulation. *J Mol Cell Cardiol* 2007 **42**: 63-70.

Domenighetti AA, Wang Q, Egger M, Richards SM, Pedrazzini T, and Delbridge LM. Angiotensin ii-mediated phenotypic cardiomyocyte remodeling leads to age-dependent cardiac dysfunction and failure. *Hypertension* 2005 **46**: 426-432.

Headrick JP, Peart J, Hack B, Flood A, and Matherne GP. Functional properties and responses to ischaemia-reperfusion in langendorff perfused mouse heart. *Exp Physiol* 2001 **86**: 703-716.

Huggins CE, Domenighetti AA, Pedrazzini T, Pepe S, and Delbridge LM. Elevated intracardiac angiotensin ii leads to cardiac hypertrophy and mechanical dysfunction in normotensive mice. *J Renin Angiotensin Aldosterone Syst* 2003 **4**: 186-190.

Livak KJ, and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. *Methods* 2001 **25**: 402-408.

Mazzolai L, Nussberger J, Aubert JF, Brunner DB, Gabbiani G, Brunner HR, and Pedrazzini T. Blood pressure independent cardiac hypertrophy induced by locally activated renin-angiotensin system. *Hypertension* 1998 **31**: 1324-1330.

Mazzolai L, Pedrazzini T, Nicoud F, Gabbiani G, Brunner HR, and Nussberger J. Increased cardiac angiotensin ii levels induce right and left ventricular hypertrophy in normotensive mice. *Hypertension* 2000 **35**: 985-991.

Mellor KM, Bell JR, Young MJ, Ritchie RH, and Delbridge LM. Myocardial autophagy activation and suppressed survival signaling is associated with insulin resistance in fructose-fed mice. *J Mol Cell Cardiol* 2011 **50**: 1035-1043.

Mellor KM, Curl CL, Chandramouli C, Pedrazzini T, Wendt IR, and Delbridge LM. Ageing-related cardiomyocyte functional decline is sex and angiotensin ii dependent. *Age (Dordr)* 2014 **36**: 9630.

Munasinghe PE, Riu F, Dixit P, Edamatsu M, Saxena P, Hamer NS, Galvin IF, Bunton RW, Lequeux S, Jones G, Lamberts RR, Emanuelli C, Madeddu P, and Katare R. Type-2 diabetes increases autophagy in the human heart through promotion of beclin-1 mediated pathway. *Int J Cardiol* 2016 **202**: 13-20.

Reichelt ME, Mellor KM, Curl CL, Stapleton D, and Delbridge LM. Myocardial glycophagy-a specific glycogen handling response to metabolic stress is accentuated in the female heart. *J Mol Cell Cardiol* 2013 **65**: 67-75.

Reichelt ME, Willems L, Peart JN, Ashton KJ, Matherne GP, Blackburn MR, and Headrick JP. Modulation of ischaemic contracture in mouse hearts: A 'supraphysiological' response to adenosine. *Exp Physiol* 2007 **92**: 175-185.

Sakaguchi K, Takeda K, Maeda M, Ogawa W, Sato T, Okada S, Ohnishi Y, Nakajima H, and Kashiwagi A. Glucose area under the curve during oral glucose tolerance test as an index of glucose intolerance. *Diabetology International* 2016 **7**: 53-58.

CHAPTER 3

Diabetes-induced diastolic dysfunction is more marked in female mice, despite less pronounced hyperglycaemia

Manuscript generated from this chapter:

Chandramouli C*, Reichelt ME*, Varma U, Curl CL, Ritchie RH, Mellor KM[#], Delbridge LMD[#]. Diabetes-induced diastolic dysfunction is more marked in female mice, despite less pronounced hyperglycaemia. *Biol Sex Differ*. [In preparation]

3.1 Introduction

Diabetes is a condition of epidemic proportions with cardiovascular mortality as the leading cause of death worldwide. Clinical and experimental findings have reported sex differences in systemic glucose dysregulation in diabetes. While men often present with increased fasting glucose (Glumer *et al.*, 2003; Sicree *et al.*, 2008), glucose intolerance, characterised by prolonged hyperglycaemia following acute glucose loading, is more prevalent in women (Dotevall *et al.*, 2007; Glumer *et al.*, 2003; Sicree *et al.*, 2008; Williams *et al.*, 2003). It has been suggested that usage of fasting blood glucose as the primary diabetic diagnostic marker leads to under-diagnosis and underrepresentation of female diabetic patients in clinical trials (Pomerleau *et al.*, 1999). In experimental settings, hyperglycaemia has been observed to be less marked in females or equivalent to males in the well-established streptozotocin (STZ) model of type 1 diabetes (T1D) using different mouse strains (Ceylan-Isik *et al.*, 2006a; b; Matsumoto *et al.*, 2008; Nuno *et al.*, 2010). Similarly, less pronounced insulin resistance and hyperglycaemia are reported in high-sucrose (Horton *et al.*, 1997) and high-fructose (Galipeau *et al.*, 2002) fed female compared to male rodents. Disturbances in lipid metabolism in a genetic T2D model (Goto-Kakizaki rat) are shown only in females (Desrois *et al.*, 2004). Thus, differences in sex-specific systemic diabetic characteristics are observed to some extent in experimental diabetic models.

Diabetic cardiomyopathy is associated with distinct structural and functional alterations in the heart independent of other risk factors, e.g. coronary artery disease or hypertension (Boudina *et al.*, 2010). Diastolic dysfunction (preceding systolic defects) in T1D and T2D (Boyer *et al.*, 2004; Zabalgaitia *et al.*, 2001) is linked with impaired relaxation, chamber stiffness and increased collagen deposition (Regan *et al.*, 1977; Shimizu *et al.*, 1993). The Framingham heart study reported a 5-fold increase in cardiovascular morbidity and mortality in diabetic women compared to a 2-fold increase in diabetic men (Kannel *et al.*, 1979), suggesting that diabetic women exhibit heightened vulnerability to heart disease. Ischemic heart disease in young T1D women is linked with high mortality (Laing *et al.*, 2003; Secrest *et al.*, 2010) while this link is not as evident in older women (Laing *et al.*, 1999). Another study involving young-adolescent T1D patients has identified significant cardiac hypertrophy and diastolic dysfunction in females but not males (Suys *et al.*, 2004). In contrast to the clinical data, some experimental studies have reported less pronounced T1D-induced cardiac

dysfunction in females relative to males. *Ex vivo* evaluation of young (6 weeks) STZ-treated rats exhibited less impairment of pressure development ($+dP/dt$) in female compared with male hearts (Rodrigues *et al.*, 1987). Other isolated heart studies in adult STZ-treated rats (12-14weeks) show left ventricular pressure development is less adversely affected in females despite similar hyperglycaemia in both sexes (Tuncay *et al.*, 2007; Yaras *et al.*, 2007). At present, experimental correlates of the increased female jeopardy observed clinically in T1D are yet to be identified.

Metabolic stress as a result of impaired glucose uptake in insulin resistant or insulin deficient settings is a key feature of diabetic cardiomyopathy. Surprisingly, glycogen deposition occurs in the diabetic myocardium of humans (Sakakibara *et al.*, 2011; Warren, 1930) and in rodent models (Alfarano *et al.*, 2011; Lajoie *et al.*, 2004; Shearer *et al.*, 2011). The basis for this paradoxical glycogen accumulation when cardiac glucose supply is restricted is not understood. Evidence of sex differences in glycogen and glucose handling from other non-pathological settings are available from experimental models. With nutrient-deprivation stress (48 hrs fasting) in mice, a female-specific cardiac glycogen accumulation associated with differential processing of glycogen was observed (Reichelt *et al.*, 2013a). A reduced utilisation of glucose in ageing female, not male, hearts was shown through FDG-PET/CT imaging in healthy human subjects (Kakinuma *et al.*, 2013). However, sex differences in the glycogen-handling responses in T1D are yet to be investigated.

Given the sex-specific differences reported in the systemic regulation of glucose and increased early occurrence of diastolic dysfunction in diabetic women, it was hypothesised that female diabetic mice will have a differential systemic and an accentuated cardiac response to diabetic stress. The aim of this study was to examine sex differences in the systemic, cardiac structural and functional pathology in STZ-induced T1D mice. This current study is the first to demonstrate that female T1D mice exhibit exacerbated diastolic and systolic despite less pronounced hyperglycaemia relative to male mice.

3.2 Specific Methodology

Animals and induction of chronic or acute insulin deficiency

Male and female mice on C57/Bl6J background (n=8-12/group) were induced with acute and chronic T1D as described in Sections 2.2 and 2.4 respectively. To induce chronic insulin deficiency, diabetes was induced at 15 weeks of age as described previously (Ritchie *et al.*, 2012) by 5 consecutive intraperitoneal injections of 55 mg/kg STZ or citrate buffer controls. Diabetes was allowed to develop for subsequent 8 weeks to investigate chronic diabetic complications. In the acute insulin deficiency study, similar injection dosage (5 x 55 mg/kg STZ) was administered at 8 weeks of age. Mice were classified as acute insulin deficiency when blood glucose levels reached 50% increase from baseline (~3-5 days post injections). Blood glucose was measured using a glucometer (ACCU-CHEK® Advantage glucometer, Roche, Mannheim, Germany) using the tail prick method.

Glucose tolerance testing

Glucose tolerance testing was conducted in mice of both sexes at 8 weeks after STZ injected following administration of 1.5 mg/kg glucose solution to 6 hours fasting as described in Section 2.5.

Tissue, blood and plasma collection

At 8 weeks after STZ treatment, mice were anaesthetised (Sodium Pentobarbital, 70 mg.kg, i.p), hearts were excised and tissue, blood and plasma samples were collected and snap-frozen in liquid N₂ as described in Section 2.6.

Insulin

Plasma insulin levels were measured using an ultrasensitive mouse insulin ELISA kit (Crystal Chem, IL, USA) as described in Section 2.7.

HbA1c measurement

Glycated haemoglobin (HbA1c) was measured using a latex agglutination inhibition immuno-assay method on a cobas 101 cartridge-based system (Roche, Basel, Switzerland) as described in detailed in Section 2.8.

***In vivo* echocardiography**

Mice were anaesthetised after 8 weeks with tribromoethanol (2.5% solution, 0.01 ml/g) and echocardiography was performed using a Vivid 9 Dimension machine (GE Vingmed, CA, USA) with a 15MHz i13L linear phased array probe as described in Section 2.13.

Histological analysis of collagen content

Picrosirius Red Staining for collagen content was performed in formalin-fixed tissues as described in Section 2.14.

Gene expression analysis

RNA extraction and reverse transcription were performed on frozen ventricular sections as mentioned in Sections 2.15 and 2.16. Metabolic and glycogen handling gene expression analysis was conducted using a customised mouse RT² PCR Array as previously described in Section 2.17.

Measurement of glycogen content

Cardiac glycogen concentrations were enzymatically measured as described previously (Mellor *et al.*, 2014; Reichelt *et al.*, 2013a). Briefly, ventricular homogenates were digested with amyloglucosidase (Roche, Applied Science, Indiana, IN, USA) at 50°C for 60 mins in 0.1 M sodium acetate, pH 6.0. Following centrifugation at 14,000g for 2 mins, the supernatant was assayed for glucose at A₄₅₀ nm using a two enzyme, colorimetric glucose assay (Sigma-Aldrich, St-Louis, MO, USA). Glycogen levels were measured in nM glycosyl units and normalised to protein (µg/µL), determined by the Lowry method (Peterson, 1977).

Statistical analysis

Data are presented as mean±SEM. Statistical analysis was performed using GraphPad Prism V7.0 (GraphPad, CA, USA). Data were analysed by two-way ANOVA and post-hoc Fisher's LSD test. A p-value of <0.05 was considered statistically significant.

3.3 Results

3.3A Female systemic phenotype is less pronounced than male in T1D

To characterise sex differences in diabetic systemic responses, glycemic and insulin status was examined in STZ-treated male and female mice. Eight weeks of STZ treatment induced significant increases in blood glucose and HbA1c levels in both sexes. The STZ-induced increase in blood glucose was more pronounced in male than female mice (Male-STZ vs. Ctrl: 2.8-fold and Female-STZ vs. Ctrl: 1.6-fold, $p < 0.05$, Figure 3.1A). This was accompanied by an STZ-induced reduction in insulin levels in males but not females. Interestingly, insulin levels in control female mice were lower than in control males (Male: 1.1 ng/mL vs. Female: 0.4 ng/mL, $p < 0.05$, Figure 3.2B). STZ-induced elevations in HbA1c levels, reflecting long-term glycemic regulation correcting for daily fluctuations in blood glucose, were more marked in males than females with T1D (Male-STZ vs. Ctrl: 86% increase and Female-STZ vs. Ctrl: 37% increase, $p < 0.05$, Figure 3.1C). Glucose tolerance tests (GTT) performed on fasted mice demonstrated slower clearance of glucose from plasma with T1D in both sexes (Figure 3.1E) and a corresponding increase in area under the GTT curve in the male and female STZ groups relative to controls (Figure 3.1F). A limitation in GTT experiments was the measurable range of the glucometer. Blood glucose measurements at 15 mins and 30 mins time points in Male STZ group potentially reported lower than actual values due to the upper limit of the glucometer, 33.3 mmol/L. However, even with this limitation causing a blunted increase in area under the curve in male T1D, sex differences in glucose tolerance are evident. Therefore, both male and female mice had increased blood glucose, HbA1c, glucose intolerance and reduced insulin levels with STZ. All of these systemic changes were less pronounced in female STZ mice.

3.3B Onset of STZ-induced systemic phenotype is delayed in females relative to males

Given the differences in systemic glycemic measures in male and female mice following the chronic STZ (8 weeks) treatment, sex differences were evaluated in an acute insulin withdrawal setting to detect changes in the early development stage of diabetes. Prior to STZ injections, there were no differences in baseline blood glucose between any groups (Figures 3.2A and 3.2B). Following five consecutive STZ injections, the time taken to reach 50% increase in blood glucose from baseline was

shorter in males than females (Male-STZ: 2 days vs. Female-STZ: 7 days, Figure 3.2A). At the timepoint of blood glucose levels presenting at 50% above baseline, plasma insulin levels were significantly lower in STZ males (Male-Ctrl: 1.7 ± 0.5 ng/mL vs. STZ: 0.4 ± 0.1 ng/mL, $p < 0.05$, Figure 3.2C) and not different in female STZ mice. Interestingly, the basal insulin levels in male controls were ~ 1.5 ng/mL higher than female vehicles (Figure 3.2C). These changes in insulin levels within 1 week of STZ were similar to that observed in the chronic diabetic setting. Thus, sex differences at early stages of STZ-induced T1D are evident, with a more rapidly developing diabetic systemic pathology in males.

3.3C Female *in vivo* cardiac diastolic dysfunction is more severe than male

To determine whether sex disparities in the systemic response of STZ related to differential cardiac diastolic functional outcomes, 4-chamber apical echocardiography was performed in male and female STZ mice at 8 weeks after STZ injections. The increase in the ratio of transmitral Doppler early filling velocity to tissue Doppler early diastolic mitral annular velocity (E/E') is used as one of the key predictors of diastolic dysfunction (Lee *et al.*, 2008). Representative traces of pulse wave (Figure 3.3A) and tissue Doppler (Figure 3.3B) suggest impaired relaxation with STZ in females. Despite the less marked increase in blood glucose, increased E/E' was evident in STZ females (Female-STZ vs. Ctrl: 53.5%, $p < 0.05$, Figure 3.3C) but not males. Ratios of early to late diastolic mitral valve tissue (E'/A') and flow (E/A ratio) velocities were both not different with STZ in both sexes (Figure 3.3D-E). Despite the less severe hyperglycaemia in diabetic females, *in vivo* cardiac diastolic dysfunction associated with STZ is more severe than male.

3.3D Mild systolic dysfunction is evident in female but not male mice

In vivo systolic function was assessed from the *m-mode* parasternal short axis echocardiographic measurements. Images from *m-mode* revealed a dilated phenotype after 8 weeks of T1D in both sexes (Figure 3.4A). Systolic function was preserved with T1D in both sexes with ejection fractions more than 60% in all groups, as observed clinically in patients with heart failure with preserved ejection fraction (Gladden *et al.*, 2014). Ejection fraction was marginally reduced in females (Female-Veh $67.5 \pm 21.5\%$ vs. STZ: $61.3 \pm 2.1\%$, $p < 0.05$, Figure 3.4B) but not in male T1D (Figure 3.4B). Similarly, fractional shortening was reduced by 4.1% in females ($p < 0.05$, Figure 3.4C)

while it remained unchanged in males (Figure 3.4C). Collectively, this suggests the onset of a mild systolic dysfunction in with STZ in female but, not male mice.

3.3E Modest dilation and ventricular wall thinning with T1D in both sexes

Anatomic changes in the heart geometry were examined by measuring septal and posterior wall thickness from *m-mode* echocardiography and cardiac weight indices. Posterior wall thickness (PWT) was moderately reduced with diabetes, and this effect was accentuated in females (Male-STZ: 11.3% vs. Female-STZ: 44.3% decrease, $p < 0.05$, Figure 3.5A). Septal wall thickness (IVSd) was reduced to similar extent with STZ in both sexes (Male-STZ: 12.8% and Female-STZ: 12.9% decrease, $p < 0.05$, Figure 3.5B). Chamber diameter was significantly larger in males at end diastole (Male-STZ vs. Ctrl: 5.2% increase, $p < 0.05$, Figure 3.5C) and females at end systole (Female-STZ: 10.6% increase, $p < 0.05$, Figure 3.5D). These structural differences were accompanied by a reduction in heart weight only in males with STZ (Male-STZ: 10.2% decrease, $p < 0.05$, Figure 3.5E), which was corrected when normalised to tibia length (Figure 3.5F). Cardiac weight indices were unchanged with diabetes in both sexes and lower in females than males in respective control and T1D comparisons (Figure 3.5F, Table 3.2). Thus, wall thinning and chamber dilation was observed in the diabetic hearts of both sexes.

3.3F Increased myocardial collagen deposition with T1D in both sexes

To determine whether the accentuated T1D female functional pathology corresponded to fibrotic structural remodelling, myocardial collagen content was assessed. Picrosirius red stained transverse ventricular sections revealed increased collagen deposition with diabetes in both sexes as depicted in the representative traces given in Figure 3.6A. Quantification of the percentage collagen normalised to total area showed significantly increased ventricular collagen content in both sexes (Male-STZ vs. Ctrl: 2-fold and Female-STZ vs. Ctrl: 1.8-fold, $p < 0.05$, Figure 3.6B) with 8 weeks of STZ. No differences were detected between males and females with STZ. Thus, fibrotic infiltration was increased with diabetes to a similar extent in both males and females.

3.3G Sex differences in glucose and glycogen handling with T1D

Previously, it has been demonstrated that female mice exhibit a more marked glycogen response to acute metabolic stress (fasting) (Reichelt *et al.*, 2013b). Thus cardiac

glycogen stores and metabolic gene expression were measured in male and female STZ mice to determine whether differential cardiac glucose metabolic dysregulation and storage abnormalities may underline the functional deficit observed in T1D female mice. Metabolic and glycogen-handling gene expression changes were assessed using RT profiling. mRNA expression was determined using the $2^{-\Delta\Delta C_t}$ calculation and the fold change values were normalised to *18S* or β -actin reference genes. Significant downregulation in *hexokinase (HK)* expression was observed in males (Male-STZ vs. Ctrl: 0.7 ± 0.1 -fold decrease, $p < 0.05$, Figure 3.7A), while it remained unchanged in females. *Glucokinase (Gck)* gene expression was significantly lower in both sexes (Male-STZ vs. Ctrl: 0.75 ± 0.01 -fold and Female-STZ vs. Ctrl: 0.53 ± 0.12 -fold decrease, $p < 0.05$, Figure 3.7A), suggesting lower flux of glucose being converted to G-6-P. *Phosphofructokinase (PFK)* expression was decreased in males (Male-STZ vs. Ctrl: 0.73 ± 0.06 -fold decrease, $p < 0.05$, Figure 3.7A) and surprisingly, increased in females (Female STZ vs. Ctrl: 1.16 ± 0.04 -fold increase, $p < 0.05$, Figure 3.7A), hinting towards potentially increased rate of glycolysis in females, not males (Figure 3.7A). When glycogen-handling genes were examined, *glycogen synthase kinase 3 β (Gsk3 β)* expression was significantly higher in females (Female STZ vs. Ctrl: 1.22 ± 0.02 -fold increase, $p < 0.05$, Figure 3.7B) while it was unchanged in males. Expression of *glycogen debranching enzyme (Agl)* and *glycogen phosphorylase (Pygm)* were both significantly higher in females (Female-STZ vs. Ctrl: *GDE*, 1.48 ± 0.06 -fold; *GP*, 1.28 ± 0.04 -fold increase, $p < 0.05$ Figure 3.7B) and not different in males. *Acid alpha-glucosidase (Gaa)* expression was unchanged in both sexes (Figure 3.7B). Thus, sex differences exist in the route of glycogen processing in diabetic hearts, with higher expression of genes involved in glycogen breakdown in female STZ. In contrast to the female-specific increase in cardiac glycogen under nutrient deprivation (Reichelt *et al.*, 2013b), there were no T1D-induced differences in glycogen levels in either sex under chronic STZ (8 weeks STZ, Figure 3.7C) or acute STZ induction (50% increase in blood glucose from baseline, ie. 2 days in males, 7 days in females, Figure 3.7D). Therefore, sex differences in metabolic regulation of glucose and glycogen handling were evident with T1D but changes in glycogen levels were not apparent.

3.4 Discussion

This study provides a novel demonstration of exacerbated female diabetic cardiopathology despite less marked hyperglycaemia and HbA1c, compared to males. Using an *in vivo* model of T1D, this investigation has shown that despite less marked increases in blood glucose, HbA1c or glucose intolerance, diastolic dysfunction and mild systolic dysfunction occur in female mice to a more severe extent than in male mice. Structural changes associated with T1D were evidenced through wall thinning, and fibrotic infiltration in both sexes. Molecular analysis revealed sex differences in expression of genes involved in glucose and glycogen substrate metabolism. Interestingly, the key regulatory enzyme of glycolysis, *phosphofructokinase (PFK)*, mRNA expression was decreased in males and unexpectedly increased in females with diabetes. An increased drive towards glycogen breakdown was also suggested in females as observed from the moderate increase in *glycogen debranching enzyme (Agl)* and *glycogen phosphorylase (Pygm)* mRNA expression with diabetes. Together, these findings suggest an increased cardiac vulnerability in female diabetic mice linked with increased glycogenolysis and glycolysis, despite less pronounced systemic changes. This study provides new insight into sex differences in the relationship between systemic and cardiac diabetic pathology.

3.4A Female systemic diabetic presentation not as marked as in males

Clinical basis for sex differences in glucose handling in diabetes has been documented with more prominent increases in fasting blood glucose in diabetic men (Glumer *et al.*, 2003; Sicree *et al.*, 2008). Few experimental studies of T1D, which have explored sex differences, have reported variable degrees of hyperglycaemia between different strains and species (Matsumoto *et al.*, 2008; Moore *et al.*, 2014; Nuno *et al.*, 2010). The present study has shown a less marked increase in blood glucose following 5 doses of 55 mg/kg STZ injections in female compared to male mice of C57Bl/6 background, comparable to reports of female hyperglycaemia observed in STZ studies using a similar mouse strain (Nuno *et al.*, 2010) and human studies (Glumer *et al.*, 2003; Sicree *et al.*, 2008). The time taken to attain a 50% increase in blood glucose from the baseline, under acute insulin withdrawal conditions, was longer in females than in males suggesting a slow-developing systemic diabetic presentation in female mice (Figure 3.2A). Similar trend was observed in other key diabetic markers, glucose intolerance

and HbA1c, a weighted average blood glucose change over the 8 weeks STZ period, confirming this sex-specific observation. Conflicting reports from the literature on systemic changes have identified STZ intervention in mice, predominantly through multiple dosage (40-125 mg/kg), to have more (Matsumoto *et al.*, 2008), less (Nuno *et al.*, 2010) or equivalent (Moore *et al.*, 2014) increases in blood glucose in females compared to males. These discrepancies may be attributable to different STZ dosage and glucose metabolic responses between various mouse strains utilised in diabetic studies (Andrikopoulos *et al.*, 2008; Berglund *et al.*, 2008).

Surprisingly, reduction in insulin levels was observed only in males in the present study. Female control mice exhibited lower basal insulin levels compared to control males, and with no effect of STZ. This could be attributed to the ELISA kit sensitivity causing limited detection of small changes in the lower end of detection range. The sex differences observed at baseline (lower in female controls vs. male) is surprising but are comparable to control levels from other studies utilising this C57/Bl6 mouse strain (Sandu *et al.*, 2005). Additional, clinical studies examining differences in insulin levels with sex and estrogen replacement therapy (ERT) have shown high insulin levels in men, lower in women without ERT and lowest in women with ERT (Ferrara *et al.*, 1995). The smaller increase in blood glucose in females with STZ may be explained by lower susceptibility of STZ-induced pancreatic β -cell injury in female mice (Paik *et al.*, 1982) and Estradiol-treated estrogen-deficient (ArKO) mice (Le May *et al.*, 2006). In a study with lower dosage of STZ (6 consecutive 35 mg/kg), female C57BL/KsJ mice exhibited equally severe insulinitis and β -cell necrosis with less severe hyperglycaemia (Leiter, 1982). Estrogen has also been shown to dampen hyperglycaemia in animal models of diabetes by decreasing hepatic glucose production and enhancing glucose transport in skeletal muscle (Louet *et al.*, 2004).

Current criteria for the diagnosis of diabetes as recommended by the Expert Committee on Diagnosis and Classification of Diabetes Mellitus are elevated fasting blood glucose (≥ 7 mmol/L), HbA1c levels ($\geq 6.5\%$), 2 hour-plasma glucose during OGTT (≥ 11.1 mmol/L) and increased random plasma glucose (≥ 11.1 mmol/L) (Expert Committee on Diagnosis, 2003). In agreement with sex differences reported clinically, with diabetic men presenting more frequently with higher blood glucose than women (Glumer *et al.*, 2003; Sicree *et al.*, 2008), this study and other studies (Nuno *et al.*, 2010) have now shown that the extent of hyperglycaemia is lower in female diabetic mice in a T1D STZ

mouse model. Therefore, the relatively subtle glycemic insult in females may not be detected when subjected to the current hallmark diagnostic thresholds test, thus warranting sex-specific diagnostic thresholds for an earlier and accurate diagnosis of diabetes.

3.4B Severe diastolic and mild systolic dysfunction in T1D females but not males

Impaired diastolic function, which precedes systolic dysfunction, (Boyer *et al.*, 2004; Zabalgaitia *et al.*, 2001) is the most common echocardiographic manifestation in T1D patients (Boyer *et al.*, 2004; Zabalgaitia *et al.*, 2001) and rodents (Huynh *et al.*, 2012; Huynh *et al.*, 2010). Given the less pronounced hyperglycaemia in female diabetic hearts, a less severe functional decline in females was hypothesised. Surprisingly, the opposite was the case. Even with a smaller extent of increase in hyperglycaemia, more marked diastolic dysfunction (increased E/E' ratio, Figure 3.3C) and a mild reduction in ejection fraction (Figure 3.4B) were observed in the female diabetic heart. A possible mechanism for this observation is that the sustained hyperglycemia increased glycation of interstitial proteins such as collagen by forming advanced glycation end products (AGE) in the extracellular matrix, resulting in a further increase in myocardial stiffness (Mellor *et al.*, 2015). Structural changes accompanying this functional decline in female hearts include modest wall thinning at diastole and increased dilation in systole (Figure 3.5). These changes in wall dimensions were accompanied by increased interstitial fibrosis infiltration in the ventricular free wall in both sexes, characteristic of diabetic patients (Chowdhry *et al.*, 2007; Frustaci *et al.*, 2000) and experimental T1D (Kato *et al.*, 2011; Li *et al.*, 2011; Meloni *et al.*, 2012). Consistent with the findings from this thesis, Bowden *et al.* also reported no sex differences in fibrotic lesions in *db/db* T2D mice (Bowden *et al.*, 2015). Work in the STZ T1D model shows that stimulation of TGF β in a hyperglycemic context promotes fibroblast collagen production, and suggests involvement of heightened angiotensin II signalling (Benazzoug *et al.*, 1998; Connelly *et al.*, 2007; Singh *et al.*, 2008). In addition, apoptotic cardiomyocyte loss is evident when there is fibrotic infiltration in both T1D and T2D experimental models (Barouch *et al.*, 2006; Fiordaliso *et al.*, 2000). Despite these evident morphological changes, cardiac weight indices, when normalised to tibia length, were unchanged. A suggested explanation is that extracellular matrix volume increases as a “backfill” response to the possible myocyte loss occurring through ventricular wall thinning (Mellor *et al.*, 2011).

Perplexingly, male functional deficit was not as apparent with STZ in this study although the morphological changes are comparable to female diabetic hearts. The reason behind this discrepancy is unclear. Perhaps, it is attributable to the trend towards a reduced heart rate with STZ in males ($p=0.08$, Table 3.1). This is a common observation in most experimental diabetic rat models (Annapurna *et al.*, 2009; Hu *et al.*, 2011; Sharma *et al.*, 2011) in contrast to normal heart rates observed in diabetic patients (Palmieri *et al.*, 2008). Inferences based on heart rate dependent flow parameters (E/A ratio) from rodent echocardiographic analysis will therefore be affected. The slower heart rate in diabetic rodents are possibly due to greater (untreated) glucose dysregulation in the absence of insulin supplementation observed in rodent models, which do not recapitulate the clinical state accurately.

The data from this chapter on accentuated female functional decline in diabetes is also consistent with earlier onset of diastolic dysfunction observed in a clinical diabetic context (Laing *et al.*, 1999; Suys *et al.*, 2004) and mouse T1D model (Moore *et al.*, 2014). Ischemic heart disease is associated with exceptionally high mortality in women at a young age (Laing *et al.*, 1999). Another study among young T1D children identified increased cardiac hypertrophy and diastolic dysfunction in female, not male children. This female jeopardy was also associated with higher glycosylated haemoglobin, suggesting greater glucose dysregulation (Suys *et al.*, 2004). Moore *et al.* have previously described an earlier onset of diastolic dysfunction in female CD1 mice after 8 weeks of STZ treatment with similar increases in blood glucose in both sexes (Moore *et al.*, 2014). Bowden *et al.* have also demonstrated an exaggerated hypertrophic and oxidative stress response in *db/db* T2D females contributing towards changes in structural pathology (Bowden *et al.*, 2015). The current study is the first to demonstrate diastolic dysfunction in females despite a lower increase in blood glucose when compared to males. This suggests that glycemic status may not accurately reflect cardiac pathology in female mice.

The findings in this thesis conform to heightened female cardiac response reported by Moore *et al.* (2014) and extend this work by showing that a less marked extent of hyperglycaemia is sufficient to cause exacerbated female cardiac structural and functional pathology. Three other studies in the literature have investigated sex differences in the T1D heart and have demonstrated contrasting results to the present findings. Isolated heart evaluations of young (6 weeks) and adult (12-14 weeks) STZ-

treated rats showed reduced impairment in left ventricular pressure development (Rodrigues *et al.*, 1987; Tuncay *et al.*, 2007; Yaras *et al.*, 2007) in females compared to males. The ambiguities between the present findings from this chapter and these reports could be attributed to differences in *in vivo* to *ex vivo* diabetic settings. The absence of neurohormonal contributions in the latter may not capture the diabetic pathology as accurately.

3.4C Sex differences in cardiac glucose and glycogen handling

Disturbances in systemic glycemic and insulinemic status elicit cardiomyocyte metabolic stress and altered glucose handling. Despite significantly reduced cardiac glucose uptake, diabetic hearts have been shown to accumulate glycogen in human (Sakakibara *et al.*, 2011; Warren, 1930) and animal models (Alfarano *et al.*, 2011; Lajoie *et al.*, 2004; Shearer *et al.*, 2011). It has been previously demonstrated that under pathological nutrient-deprivation stress, cardiac glycogen accumulation occurs selectively in female hearts (Reichelt *et al.*, 2013b). To date, a direct investigation of sex differences on glucose/glycogen regulation in an *in vivo* diabetic setting has not been reported.

In this study, downregulation of *Gck* gene expression was apparent in both sexes and is in agreement with other diabetic models reporting reduced glycolytic flux in animals and humans (Bertrand *et al.*, 2008; Garcia *et al.*, 2009; Semenza, 2011). In males, this downregulation was associated with reduced *Hexokinase (HK)* and *Phosphofructokinase (PFK)* expression. Interestingly, with T1D in females, *Hexokinase (HK)* was unchanged and *Phosphofructokinase (PFK)* was found to be upregulated, suggesting the presence of a glycolytic drive in female diabetics. This suggests basal glucose uptake utilising non-insulin dependent transporters (GLUT1) could perhaps be higher in female hearts as an adaptive response in diabetes. Alternatively, the increased glycolytic flux could be driven by substrate feed from increased glycogen breakdown. Indeed, in female STZ hearts, *glycogen phosphorylase (Pygm)* and *debranching enzyme (Agl)* was significantly higher while the expression of *glycogen synthase (Gys1)* was unchanged compared to non-diabetic controls, suggesting increased cytosolic glycogen breakdown. With the exception of reduced expression of *glycogen synthase (Gys1)*, no other differences were significant in male diabetic hearts, validating the case for a differential diabetic glycogen-handling response in females. Surprisingly, despite sex-

specific expression changes in glycolytic and glycogen-regulating genes, absolute glycogen levels were not different in both acute and chronic settings of diabetes in either sex. Differences in protein expression and post-translational modifications of these glycogen-handling genes could possibly explain this discrepancy. Accumulation of glycogen was shown to occur in NRVMs incubated in high glucose (30 mM) conditions only when insulin (10 nM) was present (Mellor *et al.*, 2014). Therefore, the STZ mouse model may not elicit pronounced cardiac glycogen pathology under basal diabetic conditions.

3.4D Limitations

Usage of an ACCU-CHEK® glucometer with maximum detection of 33mM to monitor changes in blood glucose and during glucose tolerance test was a potential limitation. Sample dilution or alternative glucometers should be considered for future studies. The acute and chronic STZ studies presented in this chapter have not been age-matched (7 and 15 weeks old at STZ injections respectively) which may be an important consideration when directly comparing the two cohorts

3.5 Conclusions and next questions

Despite the case for earlier onset of diastolic dysfunction in T1D females established in the clinical context, potential sex-specific functional differences in experimental T1D models have received minimal focus. The findings from this chapter have demonstrated that less pronounced hyperglycaemia can be associated with severe diabetic structural and functional cardiopathology in females. Molecular evaluations suggest selective upregulation of genes involved in glycolysis and glycogen breakdown in female, but not male, hearts with diabetes. However, absolute glycogen levels were unchanged in both sexes. Further evaluation of sex differences in isolated cardiac function, ischemic vulnerability and substrate utilisation is now warranted.

Given the female-specific glycogen response in non-pathological nutrient stress (Reichelt *et al.*, 2013b) and the basis for sex differences in ischemic heart disease, it was hypothesised that basal sex differences in energy storage will become more apparent with an additional ischemic stress. Therefore, the next stage of investigations evaluated glycogen content and functional recovery of male and female diabetic hearts after being subjected to *ex vivo* simulated ischemia reperfusion.

3.6 References

- Alfarano C, Suffredini S, Fantappie O, Mugelli A, Cerbai E, Manni ME, and Raimondi L.** The effect of losartan treatment on the response of diabetic cardiomyocytes to atp depletion. *Pharmacol Res* 2011 **63**: 225-232.
- Andrikopoulos S, Blair AR, Deluca N, Fam BC, and Proietto J.** Evaluating the glucose tolerance test in mice. *Am J Physiol Endocrinol Metab* 2008 **295**: E1323-1332.
- Annapurna A, Reddy CS, Akondi RB, and Rao SR.** Cardioprotective actions of two bioflavonoids, quercetin and rutin, in experimental myocardial infarction in both normal and streptozotocin-induced type i diabetic rats. *J Pharm Pharmacol* 2009 **61**: 1365-1374.
- Barouch LA, Gao D, Chen L, Miller KL, Xu W, Phan AC, Kittleson MM, Minhas KM, Berkowitz DE, Wei C, and Hare JM.** Cardiac myocyte apoptosis is associated with increased DNA damage and decreased survival in murine models of obesity. *Circulation research* 2006 **98**: 119-124.
- Benazzoug Y, Borchellini C, Labat-Robert J, Robert L, and Kern P.** Effect of high-glucose concentrations on the expression of collagens and fibronectin by fibroblasts in culture. *Exp Gerontol* 1998 **33**: 445-455.
- Berglund ED, Li CY, Poffenberger G, Ayala JE, Fueger PT, Willis SE, Jewell MM, Powers AC, and Wasserman DH.** Glucose metabolism in vivo in four commonly used inbred mouse strains. *Diabetes* 2008 **57**: 1790-1799.
- Bertrand L, Horman S, Beauloye C, and Vanoverschelde JL.** Insulin signalling in the heart. *Cardiovasc Res* 2008 **79**: 238-248.
- Boudina S, and Abel ED.** Diabetic cardiomyopathy, causes and effects. *Rev Endocr Metab Disord* 2010 **11**: 31-39.
- Bowden MA, Tesch GH, Julius TL, Rosli S, Love JE, and Ritchie RH.** Earlier onset of diabetes-induced adverse cardiac remodeling in female compared to male mice. *Obesity (Silver Spring)* 2015 **23**: 1166-1177.
- Boyer JK, Thanigaraj S, Schechtman KB, and Perez JE.** Prevalence of ventricular diastolic dysfunction in asymptomatic, normotensive patients with diabetes mellitus. *Am J Cardiol* 2004 **93**: 870-875.

Ceylan-Isik AF, LaCour KH, and Ren J. Gender disparity of streptozotocin-induced intrinsic contractile dysfunction in murine ventricular myocytes: Role of chronic activation of akt. *Clin Exp Pharmacol Physiol* 2006a **33**: 102-108.

Ceylan-Isik AF, LaCour KH, and Ren J. Sex difference in cardiomyocyte function in normal and metallothionein transgenic mice: The effect of diabetes mellitus. *J Appl Physiol (1985)* 2006b **100**: 1638-1646.

Chowdhry MF, Vohra HA, and Galinanes M. Diabetes increases apoptosis and necrosis in both ischemic and nonischemic human myocardium: Role of caspases and poly-adenosine diphosphate-ribose polymerase. *The Journal of thoracic and cardiovascular surgery* 2007 **134**: 124-131, 131 e121-123.

Connelly KA, Boyle AJ, and Kelly DJ. Angiotensin ii and the cardiac complications of diabetes mellitus. *Curr Pharm Des* 2007 **13**: 2721-2729.

Desrois M, Sidell RJ, Gauguier D, Davey CL, Radda GK, and Clarke K. Gender differences in hypertrophy, insulin resistance and ischemic injury in the aging type 2 diabetic rat heart. *J Mol Cell Cardiol* 2004 **37**: 547-555.

Dotevall A, Rosengren A, Bartnik M, Malmberg K, Ohrvik J, Simoons M, and Ryden L. Sex-related aspects on abnormal glucose regulation in patients with coronary artery disease. *Eur Heart J* 2007 **28**: 310-315.

Expert Committee on Diagnosis aCoDM. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 2003 **26 Suppl 1**: S5-20.

Ferrara A, Barrett-Connor E, Wingard DL, and Edelstein SL. Sex differences in insulin levels in older adults and the effect of body size, estrogen replacement therapy, and glucose tolerance status: The rancho bernardo study, 1984–1987. *Diabetes Care* 1995 **18**: 220-225.

Fiordaliso F, Li B, Latini R, Sonnenblick EH, Anversa P, Leri A, and Kajstura J. Myocyte death in streptozotocin-induced diabetes in rats in angiotensin ii- dependent. *Lab Invest* 2000 **80**: 513-527.

Frustaci A, Kajstura J, Chimenti C, Jakoniuk I, Leri A, Maseri A, Nadal-Ginard B, and Anversa P. Myocardial cell death in human diabetes. *Circulation research* 2000 **87**: 1123-1132.

Galipeau D, Verma S, and McNeill JH. Female rats are protected against fructose-induced changes in metabolism and blood pressure. *Am J Physiol Heart Circ Physiol* 2002 **283**: H2478-2484.

Garcia M, Pujol A, Ruzo A, Riu E, Ruberte J, Arbos A, Serafin A, Albella B, Feliu JE, and Bosch F. Phosphofructo-1-kinase deficiency leads to a severe cardiac and hematological disorder in addition to skeletal muscle glycogenosis. *PLoS Genet* 2009 **5**: e1000615.

Gladden JD, Linke WA, and Redfield MM. Heart failure with preserved ejection fraction. *Pflugers Arch* 2014 **466**: 1037-1053.

Glumer C, Jorgensen T, and Borch-Johnsen K. Prevalences of diabetes and impaired glucose regulation in a danish population: The inter99 study. *Diabetes Care* 2003 **26**: 2335-2340.

Horton TJ, Gayles EC, Prach PA, Koppenhafer TA, and Pagliassotti MJ. Female rats do not develop sucrose-induced insulin resistance. *Am J Physiol* 1997 **272**: R1571-1576.

Hu ZC, Chen YD, and Ren YH. Methylprednisolone improves microcirculation in streptozotocin-induced diabetic rats after myocardial ischemia/reperfusion. *Chin Med J (Engl)* 2011 **124**: 923-929.

Huynh K, Kiriazis H, Du XJ, Love JE, Jandeleit-Dahm KA, Forbes JM, McMullen JR, and Ritchie RH. Coenzyme q10 attenuates diastolic dysfunction, cardiomyocyte hypertrophy and cardiac fibrosis in the db/db mouse model of type 2 diabetes. *Diabetologia* 2012 **55**: 1544-1553.

Huynh K, McMullen JR, Julius TL, Tan JW, Love JE, Cemerlang N, Kiriazis H, Du XJ, and Ritchie RH. Cardiac-specific igf-1 receptor transgenic expression protects against cardiac fibrosis and diastolic dysfunction in a mouse model of diabetic cardiomyopathy. *Diabetes* 2010 **59**: 1512-1520.

Kakinuma Y, Okada S, Nogami M, and Kumon Y. The human female heart incorporates glucose more efficiently than the male heart. *Int J Cardiol* 2013 **168**: 2518-2521.

Kannel WB, and McGee DL. Diabetes and cardiovascular disease. The framingham study. *Jama* 1979 **241**: 2035-2038.

Kato T, Yamashita T, Sekiguchi A, Tsuneda T, Sagara K, Takamura M, Kaneko S, Aizawa T, and Fu LT. Angiotensin ii type 1 receptor blocker attenuates diabetes-induced atrial structural remodeling. *J Cardiol* 2011 **58**: 131-136.

Laing SP, Swerdlow AJ, Slater SD, Botha JL, Burden AC, Waugh NR, Smith AW, Hill RD, Bingley PJ, Patterson CC, Qiao Z, and Keen H. The british diabetic association cohort study, ii: Cause-specific mortality in patients with insulin-treated diabetes mellitus. *Diabet Med* 1999 **16**: 466-471.

Laing SP, Swerdlow AJ, Slater SD, Burden AC, Morris A, Waugh NR, Gatling W, Bingley PJ, and Patterson CC. Mortality from heart disease in a cohort of 23,000 patients with insulin-treated diabetes. *Diabetologia* 2003 **46**: 760-765.

Lajoie C, Calderone A, Trudeau F, Lavoie N, Massicotte G, Gagnon S, and Beliveau L. Exercise training attenuated the pkb and gsk-3 dephosphorylation in the myocardium of zdf rats. *J Appl Physiol* 2004 **96**: 1606-1612.

Le May C, Chu K, Hu M, Ortega CS, Simpson ER, Korach KS, Tsai MJ, and Mauvais-Jarvis F. Estrogens protect pancreatic beta-cells from apoptosis and prevent insulin-deficient diabetes mellitus in mice. *Proc Natl Acad Sci U S A* 2006 **103**: 9232-9237.

Lee SW, Park MC, Park YB, and Lee SK. E/e' ratio is more sensitive than e/a ratio for detection of left ventricular diastolic dysfunction in systemic lupus erythematosus. *Lupus* 2008 **17**: 195-201.

Leiter EH. Multiple low-dose streptozotocin-induced hyperglycemia and insulinitis in c57bl mice: Influence of inbred background, sex, and thymus. *Proc Natl Acad Sci U S A* 1982 **79**: 630-634.

Li Y, Ma J, Zhu H, Singh M, Hill D, Greer PA, Arnold JM, Abel ED, and Peng T. Targeted inhibition of calpain reduces myocardial hypertrophy and fibrosis in mouse models of type 1 diabetes. *Diabetes* 2011 **60**: 2985-2994.

Louet JF, LeMay C, and Mauvais-Jarvis F. Antidiabetic actions of estrogen: Insight from human and genetic mouse models. *Curr Atheroscler Rep* 2004 **6**: 180-185.

Matsumoto T, Kakami M, Kobayashi T, and Kamata K. Gender differences in vascular reactivity to endothelin-1 (1-31) in mesenteric arteries from diabetic mice. *Peptides* 2008 **29**: 1338-1346.

Mellor KM, Bell JR, Young MJ, Ritchie RH, and Delbridge LM. Myocardial autophagy activation and suppressed survival signaling is associated with insulin resistance in fructose-fed mice. *J Mol Cell Cardiol* 2011 **50**: 1035-1043.

Mellor KM, Brimble MA, and Delbridge LM. Glucose as an agent of post-translational modification in diabetes--new cardiac epigenetic insights. *Life Sci* 2015 **129**: 48-53.

Mellor KM, Varma U, Stapleton DI, and Delbridge LM. Cardiomyocyte glycophagy is regulated by insulin and exposure to high extracellular glucose. *Am J Physiol Heart Circ Physiol* 2014 **306**: H1240-1245.

Meloni M, Descamps B, Caporali A, Zentilin L, Floris I, Giacca M, and Emanuelli C. Nerve growth factor gene therapy using adeno-associated viral vectors prevents cardiomyopathy in type 1 diabetic mice. *Diabetes* 2012 **61**: 229-240.

Moore A, Shindikar A, Fomison-Nurse I, Riu F, Munasinghe PE, Ram TP, Saxena P, Coffey S, Buntun RW, Galvin IF, Williams MJ, Emanuelli C, Madeddu P, and Katare R. Rapid onset of cardiomyopathy in stz-induced female diabetic mice involves the downregulation of pro-survival pim-1. *Cardiovasc Diabetol* 2014 **13**: 68.

Nuno DW, and Lamping KG. The role of rho kinase in sex-dependent vascular dysfunction in type 1 diabetes. *Exp Diabetes Res* 2010 **2010**: 176361.

Paik SG, Michelis MA, Kim YT, and Shin S. Induction of insulin-dependent diabetes by streptozotocin. Inhibition by estrogens and potentiation by androgens. *Diabetes* 1982 **31**: 724-729.

Palmieri V, Capaldo B, Russo C, Iaccarino M, Pezzullo S, Quintavalle G, Di Minno G, Riccardi G, and Celentano A. Uncomplicated type 1 diabetes and preclinical left ventricular myocardial dysfunction: Insights from echocardiography and exercise cardiac performance evaluation. *Diabetes Res Clin Pract* 2008 **79**: 262-268.

Peterson GL. A simplification of the protein assay method of lowry et al. Which is more generally applicable. *Anal Biochem* 1977 **83**: 346-356.

Pomerleau J, McKeigue PM, and Chaturvedi N. Relationships of fasting and postload glucose levels to sex and alcohol consumption. Are american diabetes association criteria biased against detection of diabetes in women? *Diabetes Care* 1999 **22**: 430-433.

Regan TJ, Lyons MM, Ahmed SS, Levinson GE, Oldewurtel HA, Ahmad MR, and Haider B. Evidence for cardiomyopathy in familial diabetes mellitus. *J Clin Invest* 1977 **60**: 884-899.

Reichelt M, Mellor K, Stapleton D, and Delbridge L. Glycogen responses during metabolic stress are accentuated in female hearts: A role for myocardial glycophagy. *Heart, Lung and Circulation* 2013a **22**: S63.

Reichelt ME, Mellor KM, Curl CL, Stapleton D, and Delbridge LM. Myocardial glycophagy - a specific glycogen handling response to metabolic stress is accentuated in the female heart. *J Mol Cell Cardiol* 2013b **65**: 67-75.

Ritchie RH, Love JE, Huynh K, Bernardo BC, Henstridge DC, Kiriazis H, Tham YK, Sapra G, Qin C, Cemerlang N, Boey EJ, Jandeleit-Dahm K, Du XJ, and McMullen JR. Enhanced phosphoinositide 3-kinase(p110alpha) activity prevents diabetes-induced cardiomyopathy and superoxide generation in a mouse model of diabetes. *Diabetologia* 2012 **55**: 3369-3381.

Rodrigues B, and McNeill JH. Comparison of cardiac function in male and female diabetic rats. *Gen Pharmacol* 1987 **18**: 421-423.

Sakakibara M, Hirashiki A, Cheng XW, Bando Y, Ohshima K, Okumura T, Funahashi H, Ohshima S, and Murohara T. Association of diabetes mellitus with myocardial collagen accumulation and relaxation impairment in patients with dilated cardiomyopathy. *Diabetes Res Clin Pract* 2011 **92**: 348-355.

Sandu O, Song K, Cai W, Zheng F, Uribarri J, and Vlassara H. Insulin resistance and type 2 diabetes in high-fat-fed mice are linked to high glycotoxin intake. *Diabetes* 2005 **54**: 2314-2319.

Secrest AM, Becker DJ, Kelsey SF, Laporte RE, and Orchard TJ. Cause-specific mortality trends in a large population-based cohort with long-standing childhood-onset type 1 diabetes. *Diabetes* 2010 **59**: 3216-3222.

Semenza GL. Hypoxia-inducible factor 1: Regulator of mitochondrial metabolism and mediator of ischemic preconditioning. *Biochim Biophys Acta* 2011 **1813**: 1263-1268.

Sharma V, Sharma A, Saran V, Bernatchez PN, Allard MF, and McNeill JH. Beta-receptor antagonist treatment prevents activation of cell death signaling in the diabetic heart independent of its metabolic actions. *Eur J Pharmacol* 2011 **657**: 117-125.

Shearer J, Ross KD, Hughey CC, Johnsen VL, Hittel DS, and Severson DL. Exercise training does not correct abnormal cardiac glycogen accumulation in the db/db mouse model of type 2 diabetes. *Am J Physiol Endocrinol Metab* 2011 **301**: E31-39.

Shimizu M, Umeda K, Sugihara N, Yoshio H, Ino H, Takeda R, Okada Y, and Nakanishi I. Collagen remodelling in myocardia of patients with diabetes. *J Clin Pathol* 1993 **46**: 32-36.

Sicree RA, Zimmet PZ, Dunstan DW, Cameron AJ, Welborn TA, and Shaw JE. Differences in height explain gender differences in the response to the oral glucose tolerance test- the ausdiab study. *Diabet Med* 2008 **25**: 296-302.

Singh VP, Le B, Khode R, Baker KM, and Kumar R. Intracellular angiotensin ii production in diabetic rats is correlated with cardiomyocyte apoptosis, oxidative stress, and cardiac fibrosis. *Diabetes* 2008 **57**: 3297-3306.

Suys BE, Katier N, Rooman RP, Matthys D, Op De Beeck L, Du Caju MV, and De Wolf D. Female children and adolescents with type 1 diabetes have more pronounced early echocardiographic signs of diabetic cardiomyopathy. *Diabetes Care* 2004 **27**: 1947-1953.

Tuncay E, Seymen AA, Tanriverdi E, Yaras N, Tandogan B, Uluşu NN, and Turan B. Gender related differential effects of omega-3e treatment on diabetes-induced left ventricular dysfunction. *Mol Cell Biochem* 2007 **304**: 255-263.

Warren S. The effect of insulin on pathologic glycogen deposits in diabetes mellitus. *Am J Med Sci* 1930 **179** pp. 482–488.

Williams JW, Zimmet PZ, Shaw JE, de Courten MP, Cameron AJ, Chitson P, Tuomilehto J, and Alberti KG. Gender differences in the prevalence of impaired fasting glycaemia and impaired glucose tolerance in mauritius. Does sex matter? *Diabet Med* 2003 **20**: 915-920.

Yaras N, Tuncay E, Purali N, Sahinoglu B, Vassort G, and Turan B. Sex-related effects on diabetes-induced alterations in calcium release in the rat heart. *Am J Physiol Heart Circ Physiol* 2007 **293**: H3584-3592.

Zabalgoitia M, Ismaeil MF, Anderson L, and Maklady FA. Prevalence of diastolic dysfunction in normotensive, asymptomatic patients with well-controlled type 2 diabetes mellitus. *Am J Cardiol* 2001 **87**: 320-323.

Table 3.1 Echocardiographic function characteristics of T1D male and female mice.

	Male		Female	
	Ctrl	STZ	Ctrl	STZ
E wave (mm/s)	504±39	412±39	454±55	521±82
A wave (mm/s)	278±36	245±28	226±30	352±72
E' wave (mm/s)	29.0±2.2	21.8±2.5*	28.7±1.3	21.7±2.0*
A' wave (mm/s)	23.4±2.0	23.1±1.5	22.6±4.2	30.2±6.3
PHT (ms)	10.1±0.7	10.7±1.0	8.7±0.8	14.6±3.0
Dec Slope (mm/s ²)	15016±1706	11871±1680	15769±1696	12767±2108
End diastolic vol. (mL)	134±8.8	154±8.0*	121±3.5	137±5.8
Heart rate (bpm)	478±23	437±11	486±15	460±18
Stroke vol. (mL)	87.3±6.6	96.7±4.1	80.7±2.2	84.6±3.3 [#]
Cardiac output (mL/s)	0.71±0.05	0.71±0.03	0.66±0.02	0.65±0.05

Mitral valve flow velocity, early ventricular filling phase (E wave) and during atrial contraction (A wave); mitral valve tissue movement velocity, early ventricular filling phase (E' wave) and during atrial contraction (A' wave); pressure half time (PHT). Data are presented as mean ± sem (n=5-12/group). *p<0.05 vs Ctrl, [#]p<0.05 vs male, 2-way ANOVA, annotated with LSD post-hoc analyses.

Table 3.2 Systemic and Cardiac morphological characteristics of T1D male and female mice.

	Male		Female	
	Ctrl	STZ	Ctrl	STZ
Body weight (g)	30.6±0.4	27.0±0.5*	24.0±0.7 [#]	23.0±0.6 [#]
Tibia Length (mm)	17.1±0.3	17.0±0.3	17.5±0.2	17.2±0.2
Ventricular weight (mg)	130±4.0	115±3.2*	111±4.2 [#]	95.9±2.9* [#]
Heart weight/body weight (mg/g)	4.72±0.1	4.71±0.1	4.99±0.1	4.87±0.1
Heart weight/tibia length (mg/mm)	8.46±0.2	7.63±0.2*	6.84±0.2 [#]	6.44±0.2 [#]
IVSs (mm)	1.32±0.04	1.17±0.03*	1.40±0.03 [#]	1.22±0.04*
LVPWs (mm)	1.30±0.04	1.10±0.04*	1.17±0.03 [#]	0.91±0.05* [#]
RWT (ratio)	0.53±0.02	0.42±0.01*	0.51±0.01	0.40±0.01*
DWT (ratio)	0.19±0.03	0.22±0.03	0.21±0.02	0.21±0.03

Interventricular septal wall thickness at end systole (IVSs), left ventricular posterior wall thickness at end systole (LVPWs), relative wall thickness (RWT), diastolic wall thickness (DWT). Data are presented as mean ± sem (n=14-17/group). *p<0.05 vs Ctrl, [#]p<0.05 vs male, 2-way ANOVA, annotated with LSD post-hoc analyses.

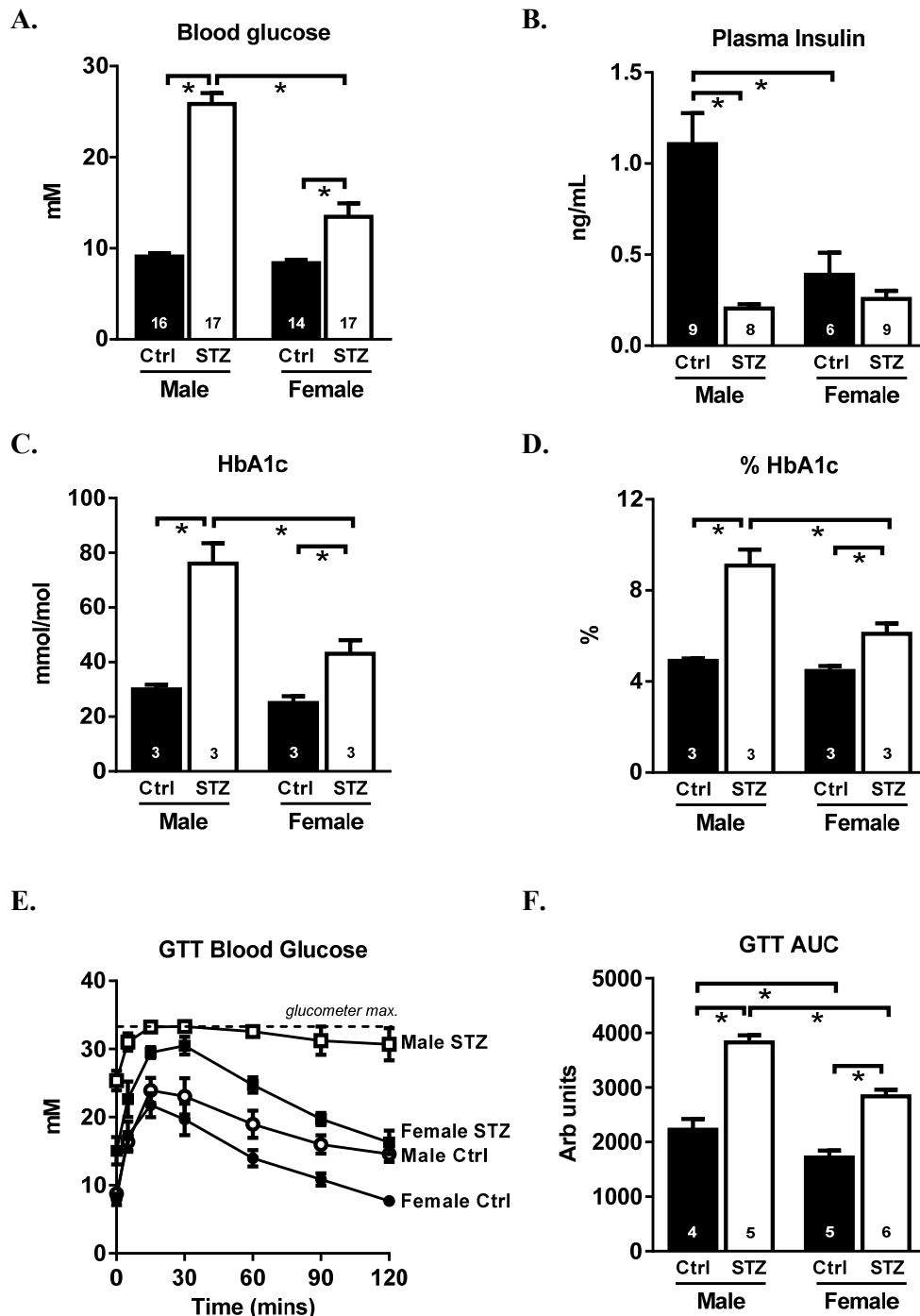


Figure 3.1. Systemic characteristics of T1D in male and female mice. **A.** Blood glucose levels. **B.** Plasma insulin measurement. **C.** Glycated haemoglobin measurement (presented as mmol/L). **D.** Glycated haemoglobin measurement (presented as %) **E.** Glucose disappearance following 1.5g/kg glucose load. Note- in some instances error bars are not discernable as they fall within symbol shapes **F.** Area under the glucose tolerance curve. Data are presented as mean $\pm$ sem. * $p < 0.05$, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

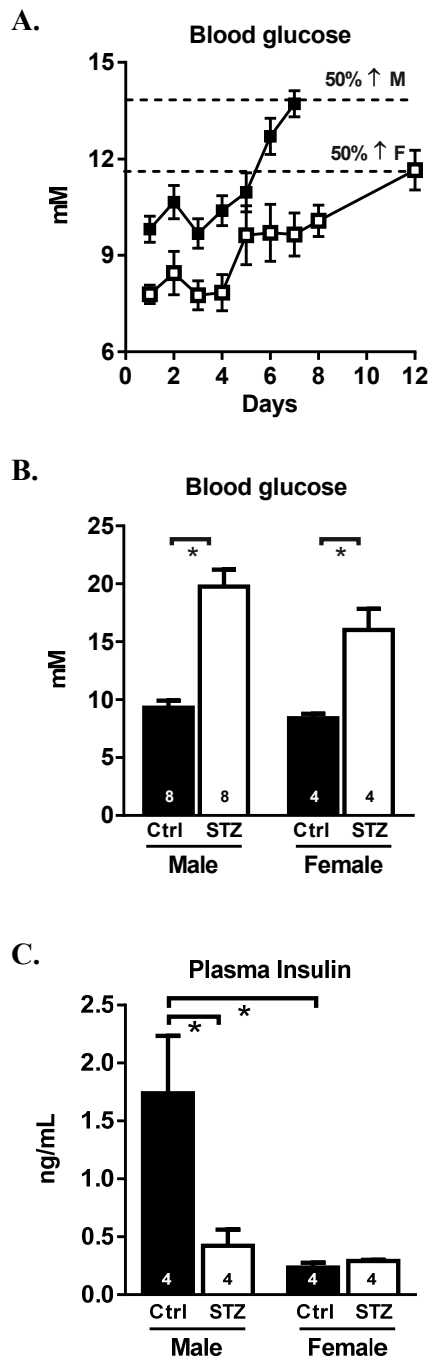


Figure 3.2. Acute systemic response to insulin withdrawal in male and female mice. **A.** Relative progression to 50% increase in blood glucose after STZ injections. **B.** Blood glucose levels. **C.** Plasma insulin levels. Data are presented as mean $\pm$ sem. * $p < 0.05$, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

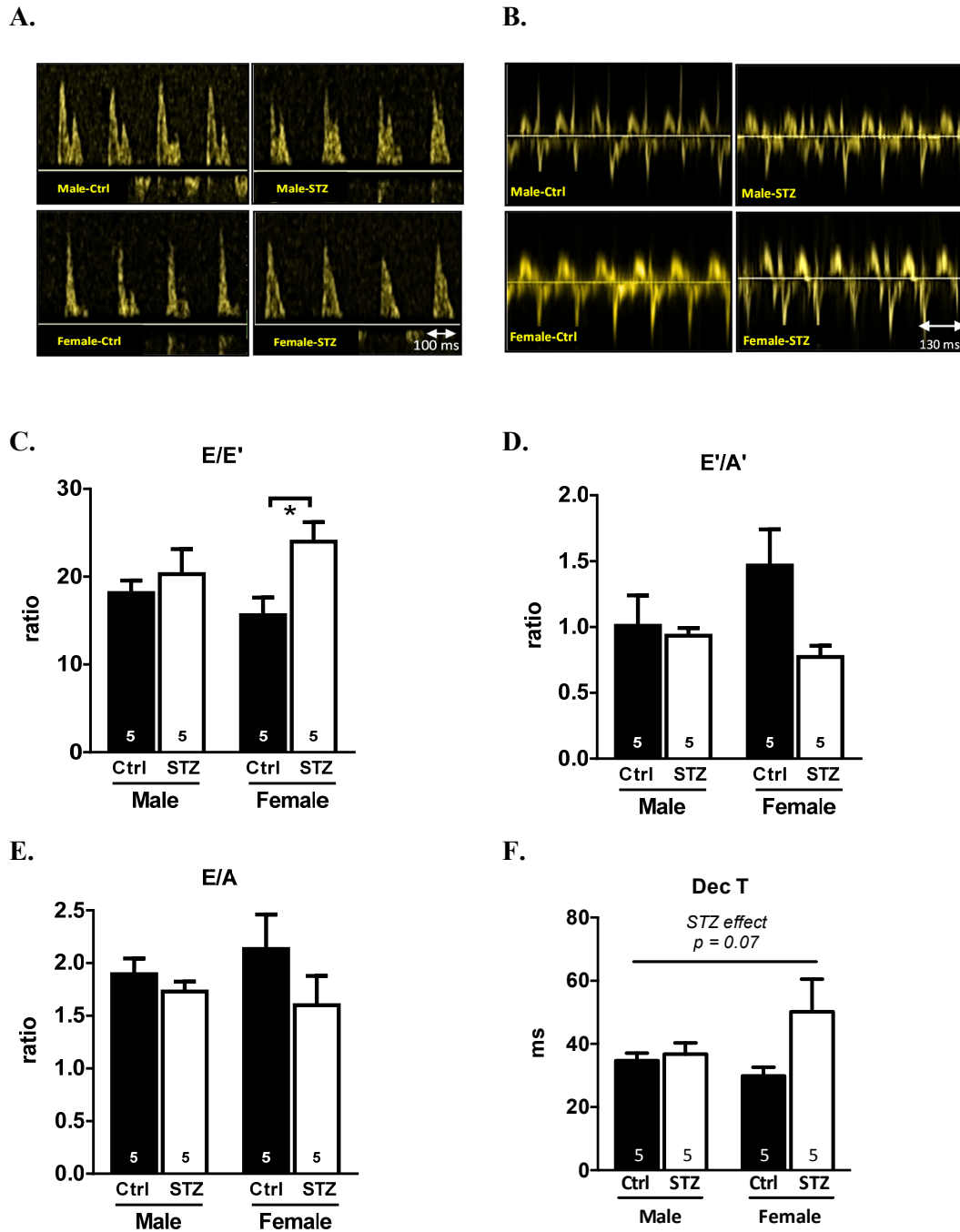


Figure 3.3. Diastolic dysfunction is more severe in T1D female mice.

A. Representative echocardiography traces from Apical-4-chamber (A4C) view of pulse-wave (blood flow) Doppler. **B.** Representative echocardiography traces from A4C view of mitral valve tissue Doppler. **C.** E/E' ratio. **D.** E'/A' ratio. **E.** E/A ratio. **F.** Mitral valve deceleration time. Data are presented as mean ± sem. * $p < 0.05$, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

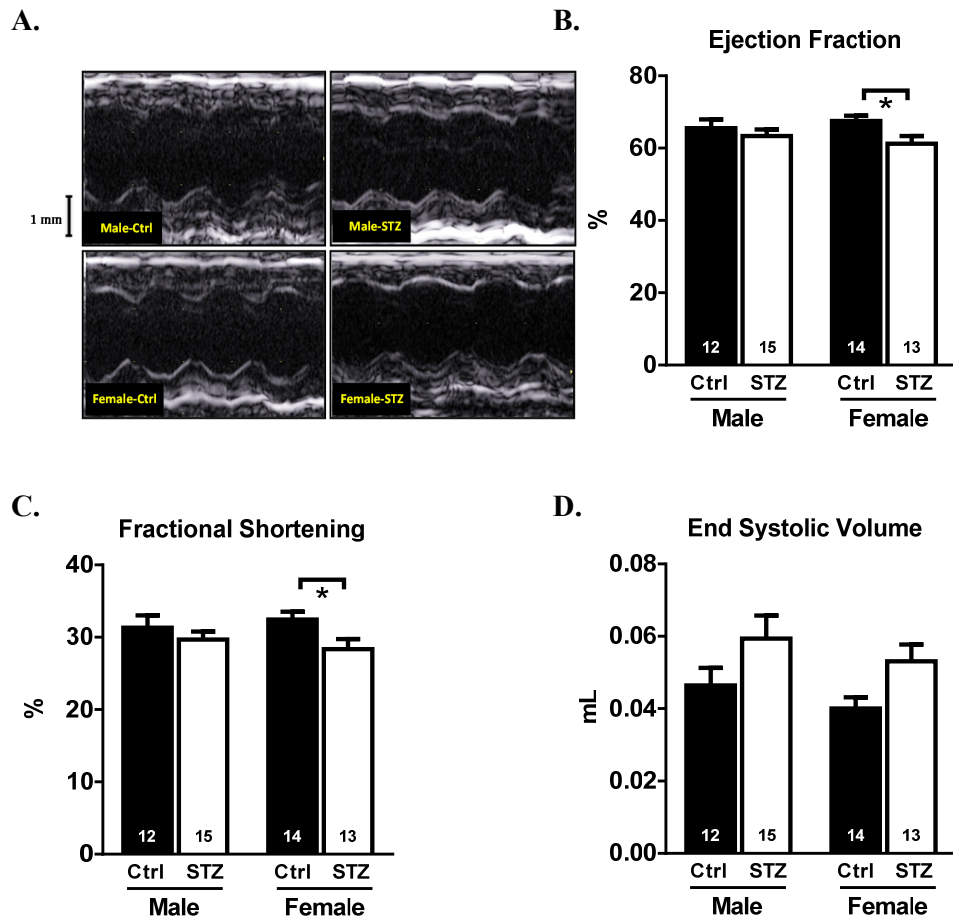


Figure 3.4. Mild systolic dysfunction in T1D female mice.

A. Representative *m-mode* traces from LV short axis view. **B.** Ejection fraction.

C. Fractional shortening. **D.** End systolic volume. Data are presented as mean $\pm$ sem.

* $p < 0.05$, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

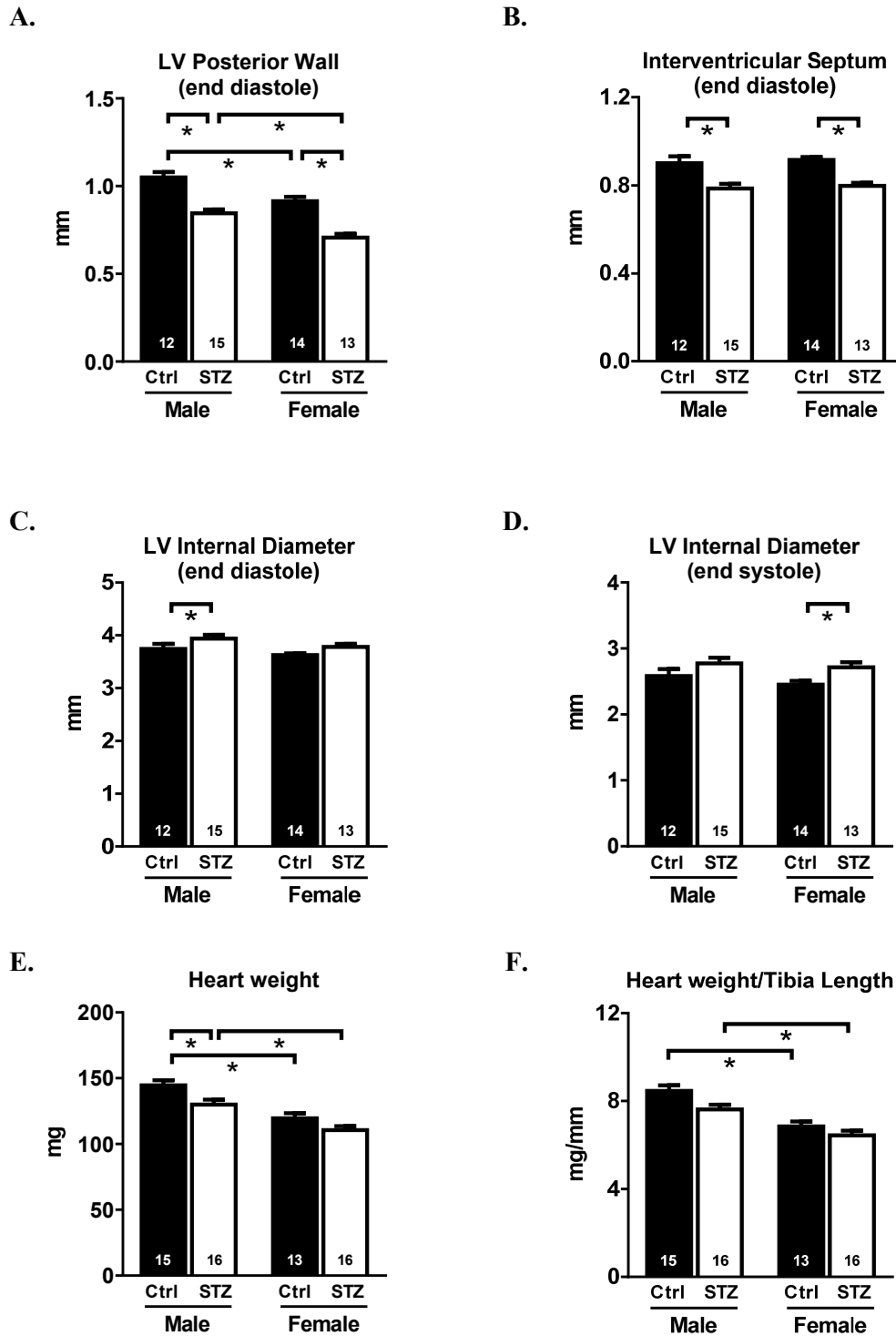
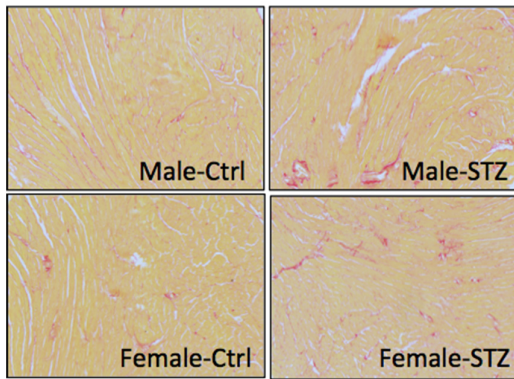


Figure 3.5. Cardiac morphological characteristics of T1D in male and female mice. **A.** Posterior wall thickness at diastole **B.** Interventricular septal wall thickness at diastole **C.** LV internal diameter at diastole **D.** LV internal diameter at systole. **E.** Cardiac weight. **F.** Cardiac weight index (heart weight (mg) normalised to tibia length (mm)). Data are presented as mean $\pm$ sem. * $p < 0.05$, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

A.



B.

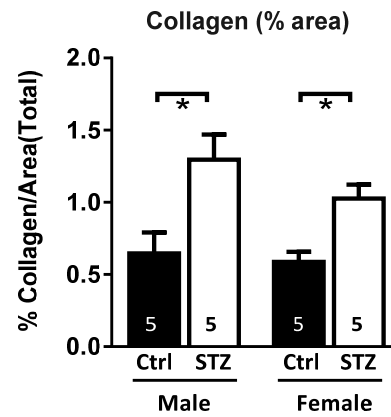
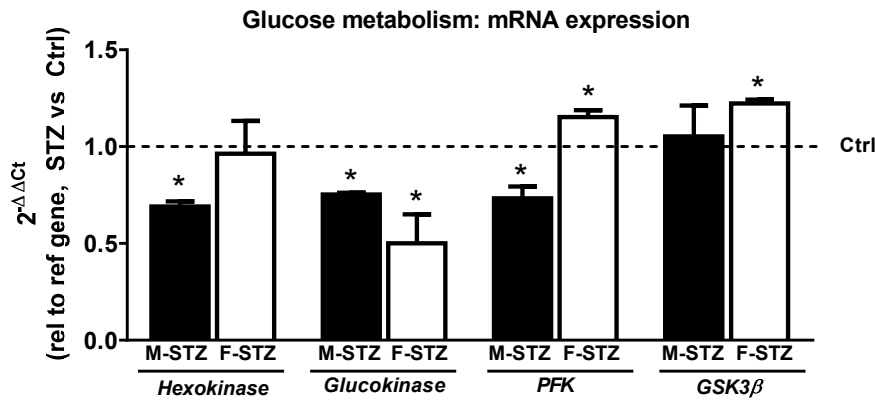


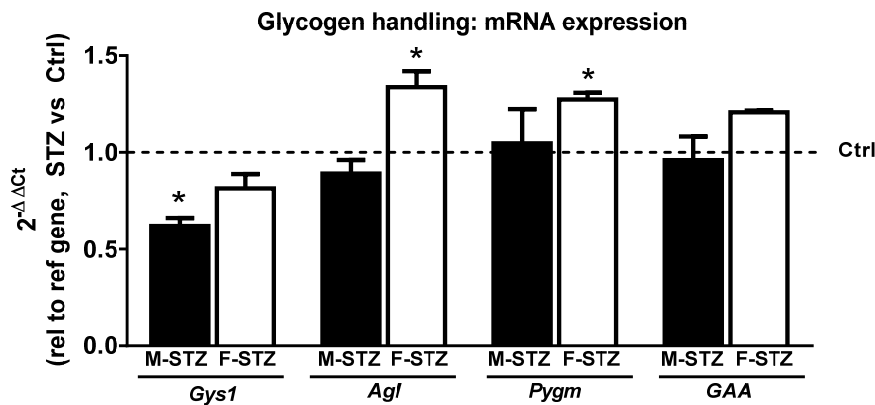
Figure 3.6. Cardiac fibrotic infiltration in T1D in male and female mice.

A. Representative picosirius red stained transverse left ventricular sections. **B.** Total collagen (as a percentage of image area) in picosirius red stained sections of ventricular tissue (n = 1-2 regions/heart; n=5 heart/group). Data are presented as mean $\pm$ sem. *p<0.05, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

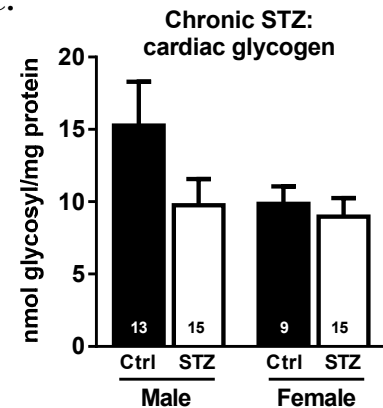
A.



B.



C.



D.

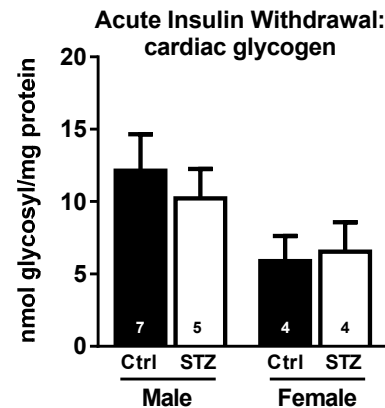


Figure 3.7. Expression of glucose-related metabolism genes and glycogen content in T1D male and female mouse hearts.

A. Glycolytic mRNA expression analysis of hexokinase, *glucokinase*, *phosphofructokinase (PFK)*, *glycogen synthase kinase 3β (GSK3β)* normalised to *18S/β-actin* reference genes ($n=3/\text{group}$) **B.** Glycogen-handling mRNA expression profile of *glycogen synthase (Gys1)*, *glycogen debranching enzyme (Agl)*, *glycogen phosphorylase (Pygm)* and *acid alpha-glucosidase (Gaa)* ($n=3/\text{group}$). **C.** Glycogen levels in chronic STZ. **D.** Glycogen content in acute insulin withdrawal setting. Data are presented as mean $\pm$ sem. * $p<0.05$ vs. ctrl, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

CHAPTER 4

Differential responses to cardiac ischemia-reperfusion injury in type 1 diabetic male and female mice

Manuscript generated from combined datasets of Chapter 4 and Chapter 6:

Chandramouli C*, Reichelt ME*, Bienvenu LA, Curl CL, Ritchie RH, Mellor KM[#],
Delbridge LMD. [#] Type-1-diabetic cardiac functional and structural pathology is
exacerbated by elevated cardiac angiotensin II in male but not female mice. *Cardiovasc
Diabetol.* [In preparation]

4.1 Introduction

Ischemic heart disease is the leading cause of death in the Western population (Finegold *et al.*, 2013). Diabetes accentuates the mortality risk of ischemic heart disease 2-3 fold (IDF, 2015). Acute myocardial ischemia-reperfusion injury is one of the major detrimental effects of ischemic heart disease. Though re-establishing blood flow to correct the ischemic environment is an apparent treatment option, the return of blood flow causes paradoxical damage to the infarct area (Yellon *et al.*, 2007). Poor post-infarct prognosis in diabetic patients, with worsened effects in diabetic women, has been documented under clinical settings worldwide (Crowley *et al.*, 2003; Fraser *et al.*, 1992; Hayes *et al.*, 2011; Lundberg *et al.*, 1997; Norhammar *et al.*, 2008; Seeman *et al.*, 1993). Thus, the clinical basis for female diabetic hearts to be prone to ischemia-reperfusion injury is fairly well established but the underlying mechanisms are not well understood.

Despite increased ischemic injury susceptibility in diabetic hearts under clinical settings, animal models of diabetes in experimental setting have shown conflicting results of more, less or no difference in the extent of cardiac damage. Most literature reports of STZ treatment in rats have shown less susceptibility to ischemia-reperfusion injury under *in vivo* (Ma *et al.*, 2006; Ravingerova *et al.*, 2003) and *ex vivo* conditions (Nawata *et al.*, 2002; Ooie *et al.*, 2003; Shi-ting *et al.*, 2010). The enhanced recovery in diabetic rodents was possibly due to increased glycogen levels, reduced rate of glycolysis and decreased clearance of protons via Na^+/H^+ exchanger in ischemic stress (Feuvray *et al.*, 1997). Some T1D studies have also shown no differences (Shi-ting *et al.*, 2010) or increased susceptibility to ischemic damage when STZ was administered at a higher dosage (70 mg/kg) (Marfella *et al.*, 2002) or in combination of alloxan (Kersten *et al.*, 2000). The inconsistency between clinical and animal studies have been attributed to usage of glucose as primary substrate in *ex vivo* setting, severity of diabetes, differences in ischemic procedure (no-flow/low-flow protocol) and the absence of *in vivo* comorbidities (Paulson, 1997; Whittington *et al.*, 2012). Therefore, accentuated ischemic jeopardy observed in diabetic patients is not always observed in experimental settings. This poses a challenge in identifying new treatment targets using intervention studies using animal models.

The clinical basis for ischemic vulnerability among diabetic women following an infarct event has been documented (Crowley *et al.*, 2003; Fraser *et al.*, 1992). Yet, to date, no

work elucidating sex differences in ischemic vulnerability of T1D hearts has been conducted. The only available literature evidence on sex differences is the study by Desrois *et al.* (2004) from a T2D model. This study demonstrated that female T2D Goto-Kakizaki rat hearts exhibited poor functional recovery after ischemia while no T2D effects were observed in males. Reduced recovery in T2D females was associated with decreased pre-ischemic glycogen levels and glucose uptake during ischemia in females. No differences in recovery were observed in male diabetic hearts with higher glycogen levels (Desrois *et al.*, 2004). It has been previously demonstrated that a metabolic stress of 48 hours fasting leads to selective glycogen accumulation and altered-phagic bulk degradation of glycogen, “*glycophagy*”, in female hearts (Reichelt *et al.*, 2013a). Sex aspects of post-ischemic recovery in T1D are yet to be explored. Yet, sex differences are evident from ischemia-reperfusion studies in non-diabetic settings. Normoglycemic female hearts generally show improved myocardial viability and functional recovery as compared to males (Bell *et al.*, 2008; Brown *et al.*, 2005; Lagranha *et al.*, 2010), but some studies have also drawn associations between estrogen supplementation and exacerbated injury (Babiker *et al.*, 2007; van Eickels *et al.*, 2003). The role of sex steroids in cardiac ischemia is yet to be clearly defined.

Epidemiological and clinical studies have convincingly shown evidence of increased susceptibility to ischemic injury in diabetic individuals. Yet, experimental studies have been divided in this regard, showing more, less or no differences in diabetic ischemic susceptibility. Lack of sex-specific studies further impedes the molecular understanding of ischemic responses in diabetic hearts. The aim of this study was to investigate whether diabetic females exhibited greater ischemic injury owing to poorer glycogen utilisation. This is the first demonstration of sex differences in cardiac responses to acute *ex vivo* ischemia-reperfusion challenges in T1D. Male diabetic hearts showed greater recovery while no differences were observed in female diabetic hearts despite earlier onset of injury. Differential glycogen utilisation and glucose handling effects were evident in male and female T1D mice.

4.2 Specific Methodology

4.2A Chronic T1D induction in mice

Male and female mice on C57Bl/6J background were utilised (n=7-9/group) for isolated heart experiments. Diabetes was induced at 15 weeks of age, as described previously and detailed in Section 2.4. Blood glucose levels were measured before injections and at 7 weeks post-diabetes induction using a glucometer (ACCU-CHEK® Advantage glucometer, Roche, Mannheim, Germany).

4.2B *Ex vivo* ischemia-reperfusion

Ex vivo heart function was assessed in both male and female C57/Bl6 mice after 8 weeks of STZ treatment (final age 25 weeks) as described previously in Section 2.7. Briefly, mice were anaesthetised with sodium pentobarbitone (70 mg/kg, i.p). Hearts were rapidly excised and placed in ice cold perfusion fluid prior to cannulation of the aorta and retrograde perfusion at a constant pressure of 80 mmHg with gassed (95% O₂, 5% CO₂) modified Krebs-Henseleit buffer containing (in mmol/L): 119 NaCl, 11 glucose, 22 NaHCO₃, 4.7 KCl, 1.2 MgCl₂, 1.2 KH₂PO₄, 0.5 EDTA, 1.75 CaCl₂ and 2.0 Na pyruvate. The left ventricle was vented with a polyethylene drain inserted through the apex of the heart and a fluid filled balloon made from polyvinyl chloride film was inserted into the left ventricle and attached to a pressure transducer (AD Instruments, Castle Hill, NSW, Australia) to measure left ventricular pressure. Coronary flow was monitored using a 2N ultrasonic flow-probe in the aortic perfusion line, connected to a T402 flow meter (Transonic Systems, Inc., Ithaca, NY). Hearts were allowed to equilibrate for 15 mins prior to data collection. Non-paced data were recorded for 5 mins, and subsequent recordings were made under paced conditions (420 beats per minute). Left ventricular end-diastolic pressure (LVEDP) was set at 5 mmHg for all hearts. Baseline functional measurements were made before subjecting hearts to a 20 mins period of global zero-flow normothermic ischemia. Hearts were then aerobically reperfused. Ventricular pacing was discontinued on induction of ischemia and resumed after 2 mins of reperfusion (Reichelt *et al.*, 2009). Peak contracture and time of onset of contracture were measured relative to LVEDP. Pacing at baseline and during reperfusion allows for direct comparison of mechanical performance under relatively standard conditions.

4.2C Tissue collection

Following reperfusion for 45 mins, hearts were removed from the apparatus, weighed and snap-frozen in liquid N₂ as described in Section 2.8.

4.2D Glycogen content analysis

Perfused hearts were homogenised in buffer (Tris-HCL 100mM pH7.0, EGTA 5mM; Sigma-Aldrich, MO, USA) (4°C, 5% w/v) containing phosphatase and proteinase inhibitors (Roche, Basel, Switzerland), as per the protein extraction protocol outlined in Section 2.9. Cardiac glycogen concentration was measured by digesting aliquots of mouse ventricular crude homogenate with and without amyloglucosidase (Sigma-Aldrich, St. Louis, MO, USA) at 50°C for 20 mins in a buffer containing 0.1 M sodium acetate pH 6.0. Following centrifugation at 14,000 g for 2 mins, an aliquot was removed and glucose levels were determined using a two-enzyme, colorimetric glucose assay (Sigma- Aldrich, St. Louis, MO, USA). Glycogen content was obtained from subtracting total (enzyme-digested) glucose from free glucose (undigested) levels. Glycogen concentrations reported were from glycosyl units (nmol) normalised to protein (mg, determined by Modified Lowry Assay (Peterson 1977) as described in Section 2.12) for comparative purposes.

4.3 Results

4.3A Basal left ventricular function in *ex vivo* hearts is not impaired by T1D

Changes in systemic blood glucose and cardiac weight indices with STZ in non-perfused hearts (Chapter 3) were reproduced in this cohort of mice (Table 4.1). After 7 weeks of STZ treatment, the extent of STZ-induced increased blood glucose was less pronounced in females (Female-STZ vs. Ctrl: 1.6-fold and Male-STZ vs. Ctrl: 2.8-fold increase, $p < 0.05$, Table 4.1). No differences in cardiac weight indices (CWI) were observed with diabetes in either sex, although CWI values were notably higher in all groups in comparison to the non-perfused hearts (Table 4.1 compared with Table 3.2). This is attributable to the usage of cell free crystalloid bicarbonate buffer with low oncotic pressure, which confers oedema to the perfused heart (Liao *et al.*, 2012).

Normoxic (baseline) contractile performance was assessed from Langendorff-perfused hearts after 8 weeks of STZ or citrate-buffer vehicle treatment in males and females. *Ex vivo* functional measurements were taken under paced setting (420 beats per min) during stabilisation period to obtain a standardised comparison with *ex vivo* post-ischemic function. After 15 mins of equilibration period, there were no STZ-induced differences in contractile performance in both sexes. Systolic function, described by left ventricular developed pressure and dP/dt_{max} was lower in female vs. male hearts (ANOVA sex factor effect, $p < 0.05$, Figure 4.1A and Figure 4.1C). Interestingly, a slower rate of relaxation was evident in both control and diabetic females and statistical significance was reached in STZ group, as observed by lower dP/dt_{min} in STZ females compared to STZ males (Female-STZ vs. Male-STZ: 37.4% lower, $p < 0.05$, Figure 4.1D). End-diastolic pressure (Figure 4.1B) and coronary flow (Figure 4.1E) were unchanged with diabetes in either sex. Heart rate during pre-pacing also did not differ between any of the groups (Figure 4.1F), confirming that the contractile changes observed were independent of intrinsic heart rate alterations. Thus, *ex vivo* basal function was preserved with STZ treatment in males and females. In general, female hearts depicted lower systolic function and slower rate of relaxation, consistent with previous reports on hypocontractile influence of estrogen in isolated cardiomyocytes (Curl *et al.*, 2003).

4.3B Despite earlier onset of contracture, ischemic injury is not different in female T1D mice

Time-compressed representative traces of STZ and control hearts of males (Figure 4.2A) and females (Figure 4.2B) show left ventricular pressure changes throughout 20 mins ischemia and early reperfusion period. Time to ischemic contracture, indicated by end-diastolic pressure reaching 15 mmHg during the 20 mins ischemic period, was not different with STZ in males (Figure 4.2C) but was significantly reduced in females (Female STZ vs. Ctrl: 27.6% earlier, $p < 0.05$, Figure 4.2C). Interestingly, peak contracture was not different with STZ treatment in either sex (Figure 4.2D). Sex differences were evident, with higher peak contracture in males (Male-Ctrl vs. Female-Ctrl: 40.3% higher, $p < 0.05$, Figure 4.2D). Overall, ischemic injury responses were different between the sexes: with diabetes, despite the earlier onset of contracture in females, the extent of injury was unchanged.

4.3C Pronounced functional rebound in male T1D but not in female T1D.

Although male T1D exhibited similar *ex vivo* basal function and ischemic injury as controls, a more pronounced rebound of systolic function was evident in male STZ throughout the 30 mins reperfusion period (Figure 4.3A and Figure 4.3C). The recovery (at 30 mins of reperfusion expressed as a percentage baseline) of both LV Developed pressure (Male Ctrl vs. STZ: 46.6 ± 7.3 vs. $75.4 \pm 5.8\%$ basal, $p < 0.05$, Figure 4.3A and Figure 4.4A) and $dP/dt_{\max}$ (Male Ctrl vs. STZ: 56.1 ± 9.0 vs. $93.7 \pm 8.0\%$ basal, $p < 0.05$, Figure 4.3A and Figure 4.4A) was significantly higher in male STZ hearts in comparison to controls (Figure 4.3A and Figure 4.4C). Additionally, diastolic recovery was also enhanced in male STZ hearts, as shown through lower LVEDP (Male Ctrl vs. STZ: 20.3 ± 4.0 vs. 7.9 ± 2.3 mmHg, $p < 0.05$, Figure 4.4B) and increased $dP/dt_{\min}$ at 30mins reperfusion (Male Ctrl vs. STZ: 30.9% increase, $p < 0.05$, Figure 4.3E and Figure 4.4D). Therefore, greater systolic and diastolic functional recovery was observed in male diabetic hearts following acute ischemia-reperfusion.

Despite the earlier onset of contracture in diabetic females, contractile functional recovery was unchanged with T1D as shown in representative traces for LV Developed pressure, $dP/dt_{\max}$ and $dP/dt_{\min}$ throughout the reperfusion period (Figure 4.3B, Figure 4.3D and Figure 4.3F). With STZ administration, no changes were observed LV Developed Pressure (Female Ctrl vs. STZ: 78.0 ± 9.4 vs. $70.3 \pm 4.6\%$ basal, Figure 4.4A) or $dP/dt_{\max}$ (Female Ctrl vs. STZ: 91.3 ± 9.0 vs. $92.0 \pm 5.1\%$ basal, Figure 4.4C) at 30 mins

reperfusion. Diastolic functional recovery post ischemia was also similar between control and STZ females. No T1D-induced differences were observed in LVEDP (Female Ctrl vs. STZ: 15.4 ± 3.3 vs. 9.5 ± 1.6 mmHg, Figure 4.4B) or dp/dt_{min} (Female Ctrl vs. STZ: 56.1 ± 9.0 vs. $93.7 \pm 8.0\%$ basal, Figure 4.4D) in females. Therefore, earlier onset of contracture observed in female diabetic hearts was not associated with any differences in the extent of injury and functional recovery in reperfusion.

4.4D Reduced cardiac glycogen content in post-ischemic male T1D but not in female T1D

To determine whether cardiac fuel utilisation under ischemic stress is different between male and female T1D, glycogen content was enzymatically measured in the hearts following reperfusion. Interestingly, differences in the extent of recovery in reperfusion in both male and female T1D was associated with altered glycogen and glucose handling. Free glucose levels were higher in T1D females (Female-STZ vs. Ctrl: 27.3% increase, $p < 0.05$, Figure 4.4A) and unchanged in T1D males (Figure 4.4A). A trend towards reduction in glycogen content was observed in male STZ (Male-STZ vs. Ctrl: 44 % reduced, $p = 0.08$, Figure 4.5B) but not in female STZ (Figure 4.5B). Sex differences were evident in glycogen content at the end of reperfusion, with higher glycogen in female compared to male STZ (Female-STZ vs. Male-STZ: 2.2-fold higher, $p < 0.05$ Figure 4.4B). Therefore, in male STZ, there were no differences in free glucose levels and a trend towards reduction in glycogen following reperfusion. In contrast, free glucose levels were increased in female diabetic mice while glycogen levels were unchanged. Together, these results suggest that pronounced functional rebound following reperfusion reduced was linked with increased glycogen utilisation in male T1D and this effect was not observed in female T1D.

4.4 Discussion

This study provides a novel demonstration of sex differences in *ex vivo* cardiac ischemic recovery responses in T1D. Epidemiological and clinical studies have convincingly shown evidence of increased susceptibility to ischemic injury in female diabetic individuals. The lack of sex-specific studies impedes molecular understanding of ischemic tolerance in diabetic hearts. Using an isolated perfused heart model, the current study assessed the extent of myocardial injury during ischemia-reperfusion in STZ-induced chronic T1D in males and females. T1D was associated with greater cardiac systolic and diastolic functional recovery following an acute 30 mins reperfusion period in male but not female mice. In females, the onset of ischemic contracture occurred earlier with diabetes, yet functional recovery at the end reperfusion was unchanged. Tissue analysis at the end of reperfusion indicated coincidental higher free glucose levels in female STZ mice, suggestive of a higher glucose uptake as an adaptive response during ischemia in female diabetic hearts. Interestingly, glycogen levels trended towards a decrease in males and were significantly higher in females with diabetes, indicative of a diminished glycogen utilisation scenario in females. The data from this chapter provides potential new insight into differential functional recovery associated with cardiac substrate utilisation in a diabetic ischemia reperfusion setting.

4.4A Diabetes is associated with greater post-ischemic functional recovery in male but not female diabetic hearts

During ischemia, the steady increase in diastolic pressure is characteristic of ischemic contracture (or “*rigor*”). While the mechanistic basis remains unknown, contracture may be a key factor in determining injury and post-ischemic outcome (Hearse *et al.*, 1977). In male diabetic mice, there were no changes in injury susceptibility, as suggested by the lack of differences observed in time of onset and peak contracture (Figure 4.2A-B). Surprisingly, greater recovery in contractile and diastolic function (Figure 4.4) was associated with a trend towards reduced glycogen levels (Figure 4.5B) in the heart following the acute 30 mins reperfusion period. Notably, pre-ischemic cardiac glycogen levels were not different with diabetes in both males and females as reported in Chapter 3 (Figure 3.7C), suggesting better utilisation of glycogen in male heart during ischemia. Though the enhanced functional recovery contrasts with clinical observations (discussed further in Section 4.4F), these results are consistent with many

male STZ-induced T1D reports which have demonstrated similar low susceptibility to ischemia-reperfusion injury in diabetic hearts under *in vivo* (Ma *et al.*, 2006; Ravingerova *et al.*, 2003) and *ex vivo* conditions (Ma *et al.*, 2006; Nawata *et al.*, 2002; Ooie *et al.*, 2003; Shi-ting *et al.*, 2010; Tani *et al.*, 1988). The greater recovery in experimental diabetic models has been suggested to be associated with increased pre-ischemic glycogen levels (Nawata *et al.*, 2002) and reduced Ca^{2+} uptake during reperfusion (Tani *et al.*, 1988). In the present study, male diabetic hearts depicted no differences in ischemic injury and greater recovery during acute reperfusion. Collectively, findings from the present study and literature suggest better substrate utilisation, particularly glycogen, during ischemia as a possible explanation for this greater recovery.

4.4B Despite earlier onset of contracture, extent of injury is not different in female diabetic hearts

An earlier onset and/or higher amplitude of contracture is generally indicative of an unfavourable post-ischemic outcome (Hearse *et al.*, 1977). Unlike in the males, earlier onset of contracture was evident in T1D females (Figure 4.2A), suggesting escalated ATP depletion and earlier onset of acidosis (Bell *et al.*, 2011a). These results are consistent with worsened post-infarct outcomes in diabetic women observed clinically (Ouhoumane *et al.*, 2009). Comparable ischemic injury in T1D has been shown in more severe experimental T1D male studies, which utilise a higher dosage of STZ (70 mg/kg) (Jones *et al.*, 1999; Kersten *et al.*, 2000; Marfella *et al.*, 2002). Larger infarct sizes were also reported in *in vivo* non-recovery ischemia in rats given a higher dosage of STZ (70 mg/kg) (Marfella *et al.*, 2002). Some indication of escalated injury onset in female diabetes was present in the current study.

Literature evidence of sex-specific Ca^{2+} handling responses in diabetes is available from non-ischemic studies. Therefore, a female-specific regulation in Ca^{2+} handling in during ischemic stress in T1D was expected. During ischemia, depletion of intracellular ATP levels and Ca^{2+} overload are associated with a steady increase in diastolic pressure (Reichelt *et al.*, 2007). However, in the current study, no T1D-induced differences in peak contracture (magnitude of increase in diastolic pressure) were observed at the end of ischemia in either sex (Figure 4.2B), indicating Ca^{2+} overload was not exacerbated with T1D in the cytosol. Consistent with the lack of Ca^{2+} overload with STZ in females

from this study, altered Ca^{2+} signalling has been reported to be less pronounced in *db/db* females than males (Pereira *et al.*, 2006). However, the marked Ca^{2+} changes reported in *db/db* males (Pereira *et al.*, 2006) conflicts with the findings from males in the current STZ study, possibly attributable to differences in T1D and T2D Ca^{2+} misappropriation.

In addition, contractile function measured following an acute reperfusion period was not different between female controls and diabetic mice (Figure 4.4). An accelerated rate of contracture onset was observed in female diabetics, indicative of some injury, but the extent of injury and functional recovery after acute reperfusion was similar to non-diabetic mice. Whether exacerbated functional detriment would emerge after longer periods of reperfusion is yet to be investigated.

4.4C Sex differences in post-ischemic functional recovery

In the control groups, the female contractile recovery in reperfusion was greater than in male mice (Figure 4.4). These sex differences are in agreement with studies demonstrating lower susceptibility to post-ischemic contractile deficit and cell death in females compared to males (Bell *et al.*, 2008; Brown *et al.*, 2005; Lagranha *et al.*, 2010). This estrogen-modulated “protection” is lost in association with other cardiovascular pathologies such as hypertrophy (Bell *et al.*, 2008). In the current study, with diabetes, males showed greater recovery (*vs.* Male-Ctrl) while females showed no differences, despite indications of early injury onset (*vs.* Female-Ctrl). Findings from the current study are somewhat consistent with the reports from Desrois and colleagues (2004), the only other study in the literature, which has examined sex differences in diabetic ischemic recovery. While contracture was not reported, they found that female Goto-Kakizaki (T2D) rats had 30% lower contractile functional recovery compared to their male counterparts after a 32 mins low-flow ischemia and 32 mins reperfusion protocol. These findings were associated with hypertrophy, greater insulin resistance and reduced insulin-stimulated glucose uptake in females (Desrois *et al.*, 2004). In summary, male and female diabetic mice respond differently to acute ischemia-reperfusion in diabetes. During reperfusion, functional recovery was greater in male T1D mice and unchanged in female T1D mice, despite the earlier onset of ischemic injury in the latter.

4.4D Evidence of T1D female-specific substrate handling in reperfusion

Studies reporting conferred protection in ischemia in diabetes have attributed the greater recovery in diabetic hearts to increased pre-ischemic glycogen content (Nakao *et al.*, 1993; Nawata *et al.*, 2002). Diabetic rats are also pre-conditioned (*vs.* controls), suggesting that metabolic adaptations of the myocardium during ischemia is associated with the extent of injury which transpires during subsequent reperfusion (Liu *et al.*, 1993). Glycogen increases selectively in females in the non-pathological nutrient deprivation stress of fasting (Reichelt *et al.*, 2013a). Glycogen and glucose handling differences investigated in hearts at the end of the acute reperfusion gives some intuitive molecular insights into the different ischemic responses in male and female diabetes. Interestingly, the extent of injury during acute ischemic-reperfusion in females was coincident with higher free glucose availability and no change in glycogen in the myocardium post-reperfusion (Figure 4.5A). This is suggestive of higher basal glucose uptake in female STZ mice, independent of insulin availability, when coronary circulation was reinstated in reperfusion. Estrogen is known to directly modulate insulin signalling by regulating the P13K/Akt pathway by binding to membrane-associated estrogen receptors to activate P13K (Gao *et al.*, 2002; Patten *et al.*, 2004). This could partially explain the lack of difference in functional recovery in female hearts despite an earlier onset of injury.

Although no changes were observed in pre-ischemic glycogen in acute and chronic STZ (Figure 3.3E-F), there was a trend towards reduced glycogen in male diabetic hearts following 30 mins of acute reperfusion ($p=0.08$). The enhanced recovery in males could therefore be due to better utilisation of this fuel as an energy source during ischemic stress. In contrast, females STZ mice (*vs.* Ctrl) had no differences in glycogen. Notably, female hearts also contained significantly higher glycogen levels compared to male diabetics (Figure 4.5B). This indicates diminished glycogen utilisation conditions in female STZ mice. Therefore, there are apparent sex differences in metabolic management of glucose and glycogen in diabetic hearts following acute ischemia-reperfusion. Whether this metabolic alteration is an adaptive/maladaptive response remains to be investigated.

4.4E Unchanged *ex vivo* baseline function with diabetes in both sexes

Ex vivo baseline function was not different with chronic STZ administration (Figure 4.1). The discrepancy between the *ex vivo* function and *in vivo* assessment reported in Chapter 3 is possibly due to isolated heart experimental *ex vivo* set up. Although relaxation parameters can be accurately assessed in *ex vivo* settings, it is difficult to establish the presence of diastolic dysfunction under baseline conditions as the left ventricular balloon is inflated to yield an end-diastolic pressure of 5 mmHg during the stabilisation period. This standardises the balloon pressure on the ventricular wall at diastole which allows accurate assessment of ventricular performance between different hearts of varying ventricular chamber sizes (Reichelt *et al.*, 2007). T1D-induced changes in systolic function were not detected in *ex vivo* setting in either sex. Basal sex differences were observed with female control (vs. male control) hearts exhibiting lower contractile function and a slower relaxation rate (Figure 4.1). This was in line with estrogen-mediated downregulation in cardiomyocyte performance, which has been shown in many instances in normoxia and normoglycemia (Bell *et al.*, 2011b; Curl *et al.*, 2003; Curl *et al.*, 2001). Given that no diabetes-induced differences in baseline cardiac performance were detected in this experimental *ex vivo* setting, diabetes and sex effects detected at the end of reperfusion are entirely attributable to cardiac responses during the ischemia-reperfusion period.

4.4F Limitations-Discrepancy between clinical and experimental settings

The greater reperfusion recovery following ischemia observed in male T1D in this study and other experimental studies does not correlate with higher clinical risk of mortality in diabetic patients, post-infarct (Crowley *et al.*, 2003; Hayes *et al.*, 2011; Norhammar *et al.*, 2008). The discrepancy between experimental and clinical findings could account for limitations in substrate availability and ischemic conditions used in *ex vivo* settings. Firstly, usage of Krebs-Henseleit perfusion buffer with glucose as the sole energy substrate may not be closely reflective of an *in vivo* substrate pool, which is predominantly fatty acid dependent (Paulson, 1997; Whittington *et al.*, 2012). Addition of pyruvate or free fatty acids to the Langendorff rig is avoided in this study as it presents frothing issues when sintered glass oxygenators are used to oxygenate the buffer (Bell *et al.*, 2011b). Yet, it may be key to best reflect the diabetic *in vivo* substrate environment. Indeed, reduced reperfusion function was reported in aged male

T2D Goto-Kakizaki rats with fatty acids and glucose as substrates in the perfusate (Desrois *et al.*, 2010). Secondly, reperfusion recovery measured in an acute 30 mins setting may not be comparable to long-term post-infarct recovery. Follow up of myocardial infarct patients showed no diabetes influence on 30-day mortality rate but a significantly higher 1-year and 5-year mortality rate (Ouhoummene *et al.*, 2009). Lastly, some experimental T1D studies have also shown increased ischemic damage when STZ was administered at a higher dosage (70 mg/kg) (Marfella *et al.*, 2002) or in combination with alloxan (Kersten *et al.*, 2000) or was subjected to longer duration reperfusion (2 hours) (Marfella *et al.*, 2002). It is worth noting that these studies have measured myocardial infarct size in ischemia, as opposed to functional evaluations during reperfusion (Kersten *et al.*, 2000; Marfella *et al.*, 2002). Collectively, these studies suggested that, (1) availability of glucose as the only substrate in the perfusate, (2) reperfusion functional assessment in acute, not chronic, settings and (3) less severe diabetic phenotypes could explain the enhanced recovery observed in this study. In addition, other reperfusion injury may involve ventricular fibrillation (VF), ventricular tachycardia (VT) and LDH release with loss in sarcolemmal integrity but these were not measured in this study. Therefore, findings from this study have identified important sex differences in the immediate response to ischemia in diabetes, but extrapolation of these findings to long-term outcomes is limited.

4.5 Conclusions and next questions

This investigation provides the first evidence of sex-specific responses to ischemia and functional recovery during acute reperfusion in an experimental T1D setting. Male T1D mice exhibited greater recovery in acute reperfusion and were associated with more efficient utilisation of glycogen stores. Despite the earlier onset of ischemic contracture in T1D females, the subsequent functional recovery at the end of acute reperfusion was unchanged. These findings provide new information about underlying sex-specific ischemia-reperfusion responses in the diabetic heart. Further investigation into energy utilisation, extent of cell death and fibrotic infiltration is now warranted.

This chapter extended the work presented in Chapter 3 in non-perfused hearts, where less pronounced systemic changes were associated with more severe diastolic dysfunction and structural pathology in T1D females. Tissue analysis suggested sex-specific perturbations in glucose and glycogen handling. This study examined whether

these sex differences in metabolic and functional adaptations in T1D become more apparent in an ischemic environment, which conferred an additional nutrient challenge to the diabetic myocardium. An isolated heart model was utilised to assess ischemic damage and function after an acute reperfusion period in T1D male and female hearts. In females, the functional deficit was not different with T1D (even with earlier onset of contracture). Interestingly, female T1D hearts also had higher free glucose stores and unaltered glycogen stores, suggesting diminished access to glycogen stores. These novel findings suggest sex-specific glycolytic fuel management during ischemic reperfusion, which may be important in determining the extent of underpinning injury in the heart.

Since the first report from Mellor *et al.* on increased cardiac autophagy, a programmed cell death process (Mellor *et al.*, 2011), in T2D, there has been a deluge of experimental studies examining autophagy in diabetic hearts. Sexual dimorphism has also been reported in macro-and glyco-phagic processes in non-diabetic setting (Reichelt *et al.*, 2013b). Given the sex differences in structure, function and metabolic differences in Chapter 3 and 4, the next stage of investigation (Chapter 5) therefore evaluated an operational snapshot of cell death and fuel metabolism in male and female T1D mice.

4.6 References

- Babiker FA, Lips DJ, Delvaux E, Zandberg P, Janssen BJ, Prinzen F, van Eys G, Grohe C, and Doevendans PA.** Oestrogen modulates cardiac ischaemic remodelling through oestrogen receptor-specific mechanisms. *Acta Physiol (Oxf)* 2007 **189**: 23-31.
- Bell JR, Mellor KM, Wollermann AC, and Delbridge LM.** Cardiac ischaemic stress: Cardiomyocyte Ca^{2+} , sex and sex steroids. *Clin Exp Pharmacol Physiol* 2011a **38**: 717-723.
- Bell JR, Mellor KM, Wollermann AC, Ip WT, Reichelt ME, Meachem SJ, Simpson ER, and Delbridge LM.** Aromatase deficiency confers paradoxical postischemic cardioprotection. *Endocrinology* 2011b **152**: 4937-4947.
- Bell JR, Porrello ER, Huggins CE, Harrap SB, and Delbridge LM.** The intrinsic resistance of female hearts to an ischemic insult is abrogated in primary cardiac hypertrophy. *Am J Physiol Heart Circ Physiol* 2008 **294**: H1514-1522.
- Brown DA, Lynch JM, Armstrong CJ, Caruso NM, Ehlers LB, Johnson MS, and Moore RL.** Susceptibility of the heart to ischaemia-reperfusion injury and exercise-induced cardioprotection are sex-dependent in the rat. *J Physiol* 2005 **564**: 619-630.
- Crowley A, Menon V, Lessard D, Yarzebski J, Jackson E, Gore JM, and Goldberg RJ.** Sex differences in survival after acute myocardial infarction in patients with diabetes mellitus (worchester heart attack study). *Am Heart J* 2003 **146**: 824-831.
- Curl CL, Wendt IR, Canny BJ, and Kotsanas G.** Effects of ovariectomy and 17 beta-oestradiol replacement on $[\text{Ca}^{2+}]_i$ in female rat cardiac myocytes. *Clin Exp Pharmacol Physiol* 2003 **30**: 489-494.
- Curl CL, Wendt IR, and Kotsanas G.** Effects of gender on intracellular $[\text{Ca}^{2+}]$ in rat cardiomyocytes. *Pflugers Arch* 2001 **441**: 709-716.
- Desrois M, Clarke K, Lan C, Dalmasso C, Cole M, Portha B, Cozzone PJ, and Bernard M.** Upregulation of enos and unchanged energy metabolism in increased susceptibility of the aging type 2 diabetic gk rat heart to ischemic injury. *Am J Physiol Heart Circ Physiol* 2010 **299**: H1679-1686.
- Desrois M, Sidell RJ, Gauguier D, Davey CL, Radda GK, and Clarke K.** Gender differences in hypertrophy, insulin resistance and ischemic injury in the aging type 2 diabetic rat heart. *J Mol Cell Cardiol* 2004 **37**: 547-555.

Feuvsray D, and Lopaschuk GD. Controversies on the sensitivity of the diabetic heart to ischemic injury: The sensitivity of the diabetic heart to ischemic injury is decreased. *Cardiovasc Res* 1997 **34**: 113-120.

Finegold JA, Asaria P, and Francis DP. Mortality from ischaemic heart disease by country, region, and age: Statistics from world health organisation and united nations. *Int J Cardiol* 2013 **168**: 934-945.

Fraser GE, Strahan TM, Sabate J, Beeson WL, and Kissinger D. Effects of traditional coronary risk factors on rates of incident coronary events in a low-risk population. The adventist health study. *Circulation* 1992 **86**: 406-413.

Gao F, Gao E, Yue TL, Ohlstein EH, Lopez BL, Christopher TA, and Ma XL. Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: The roles of pi3-kinase, akt, and endothelial nitric oxide synthase phosphorylation. *Circulation* 2002 **105**: 1497-1502.

Hayes AJ, Leal J, Kelman CW, and Clarke PM. Risk equations to predict life expectancy of people with type 2 diabetes mellitus following major complications: A study from western australia. *Diabet Med* 2011 **28**: 428-435.

Hearse DJ, Garlick PB, and Humphrey SM. Ischemic contracture of the myocardium: Mechanisms and prevention. *Am J Cardiol* 1977 **39**: 986-993.

IDF. Idf diabetes atlas. Brussels, Belgium: International Diabetes Federation, 2015.

Jones SP, Girod WG, Granger DN, Palazzo AJ, and Lefer DJ. Reperfusion injury is not affected by blockade of p-selectin in the diabetic mouse heart. *Am J Physiol* 1999 **277**: H763-769.

Kersten JR, Toller WG, Gross ER, Pagel PS, and Warltier DC. Diabetes abolishes ischemic preconditioning: Role of glucose, insulin, and osmolality. *Am J Physiol Heart Circ Physiol* 2000 **278**: H1218-1224.

Lagranha CJ, Deschamps A, Aponte A, Steenbergen C, and Murphy E. Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection in females. *Circ Res* 2010 **106**: 1681-1691.

Liao R, Podesser BK, and Lim CC. The continuing evolution of the langendorff and ejecting murine heart: New advances in cardiac phenotyping. *Am J Physiol Heart Circ Physiol* 2012 **303**: H156-167.

Liu Y, Thornton JD, Cohen MV, Downey JM, and Schaffer SW. Streptozotocin-induced non-insulin-dependent diabetes protects the heart from infarction. *Circulation* 1993 **88**: 1273-1278.

Lundberg V, Stegmayr B, Asplund K, Eliasson M, and Huhtasaari F. Diabetes as a risk factor for myocardial infarction: Population and gender perspectives. *J Intern Med* 1997 **241**: 485-492.

Ma G, Al-Shabrawey M, Johnson JA, Datar R, Tawfik HE, Guo D, Caldwell RB, and Caldwell RW. Protection against myocardial ischemia/reperfusion injury by short-term diabetes: Enhancement of vegf formation, capillary density, and activation of cell survival signaling. *Naunyn Schmiedebergs Arch Pharmacol* 2006 **373**: 415-427.

Marfella R, D'Amico M, Di Filippo C, Piegari E, Nappo F, Esposito K, Berrino L, Rossi F, and Giugliano D. Myocardial infarction in diabetic rats: Role of hyperglycaemia on infarct size and early expression of hypoxia-inducible factor 1. *Diabetologia* 2002 **45**: 1172-1181.

Mellor KM, Bell JR, Young MJ, Ritchie RH, and Delbridge LM. Myocardial autophagy activation and suppressed survival signaling is associated with insulin resistance in fructose-fed mice. *J Mol Cell Cardiol* 2011 **50**: 1035-1043.

Nakao M, Matsubara T, and Sakamoto N. Effects of diabetes on cardiac glycogen metabolism in rats. *Heart Vessels* 1993 **8**: 171-175.

Nawata T, Takahashi N, Ooie T, Kaneda K, Saikawa T, and Sakata T. Cardioprotection by streptozotocin-induced diabetes and insulin against ischemia/reperfusion injury in rats. *J Cardiovasc Pharmacol* 2002 **40**: 491-500.

Norhammar A, Stenestrand U, Lindback J, Wallentin L, Register of I, and Knowledge about Swedish Heart Intensive Care A. Women younger than 65 years with diabetes mellitus are a high-risk group after myocardial infarction: A report from the swedish register of information and knowledge about swedish heart intensive care admission (riks-hia). *Heart* 2008 **94**: 1565-1570.

Ooie T, Takahashi N, Nawata T, Arikawa M, Yamanaka K, Kajimoto M, Shinohara T, Shigematsu S, Hara M, Yoshimatsu H, and Saikawa T. Ischemia-

induced translocation of protein kinase c-epsilon mediates cardioprotection in the streptozotocin-induced diabetic rat. *Circ J* 2003 **67**: 955-961.

Ouhoumane N, Abdous B, Emond V, and Poirier P. Impact of diabetes and gender on survival after acute myocardial infarction in the province of quebec, canada--a population-based study. *Diabet Med* 2009 **26**: 609-616.

Patten RD, Pourati I, Aronovitz MJ, Baur J, Celestin F, Chen X, Michael A, Haq S, Nuedling S, Grohe C, Force T, Mendelsohn ME, and Karas RH. 17beta-estradiol reduces cardiomyocyte apoptosis in vivo and in vitro via activation of phospho-inositide-3 kinase/akt signaling. *Circ Res* 2004 **95**: 692-699.

Paulson DJ. The diabetic heart is more sensitive to ischemic injury. *Cardiovasc Res* 1997 **34**: 104-112.

Pereira L, Matthes J, Schuster I, Valdivia HH, Herzig S, Richard S, and Gomez AM. Mechanisms of [ca²⁺]_i transient decrease in cardiomyopathy of db/db type 2 diabetic mice. *Diabetes* 2006 **55**: 608-615.

Ravingerova T, Neckar J, and Kolar F. Ischemic tolerance of rat hearts in acute and chronic phases of experimental diabetes. *Mol Cell Biochem* 2003 **249**: 167-174.

Reichelt M, Mellor K, Stapleton D, and Delbridge L. Glycogen responses during metabolic stress are accentuated in female hearts: A role for myocardial glycophagy. *Heart, Lung and Circulation* 2013a **22**: S63.

Reichelt ME, Mellor KM, Curl CL, Stapleton D, and Delbridge LM. Myocardial glycophagy-a specific glycogen handling response to metabolic stress is accentuated in the female heart. *J Mol Cell Cardiol* 2013b **65**: 67-75.

Reichelt ME, Willems L, Hack BA, Peart JN, and Headrick JP. Cardiac and coronary function in the langendorff-perfused mouse heart model. *Exp Physiol* 2009 **94**: 54-70.

Reichelt ME, Willems L, Peart JN, Ashton KJ, Matherne GP, Blackburn MR, and Headrick JP. Modulation of ischaemic contracture in mouse hearts: A 'supraphysiological' response to adenosine. *Exp Physiol* 2007 **92**: 175-185.

Seeman T, Mendes de Leon C, Berkman L, and Ostfeld A. Risk factors for coronary heart disease among older men and women: A prospective study of community-dwelling elderly. *Am J Epidemiol* 1993 **138**: 1037-1049.

Shi-ting W, Mang-hua X, Wen-ting C, Feng-hou G, and Zhu-ying G. Study on tolerance to ischemia-reperfusion injury and protection of ischemic preconditioning of type 1 diabetes rat heart. *Biomed Pharmacol* 2010

Tani M, and Neely JR. Hearts from diabetic rats are more resistant to in vitro ischemia: Possible role of altered Ca^{2+} metabolism. *Circ Res* 1988 **62**: 931-940.

van Eickels M, Patten RD, Aronovitz MJ, Alsheikh-Ali A, Gostyla K, Celestin F, Grohe C, Mendelsohn ME, and Karas RH. 17-beta-estradiol increases cardiac remodeling and mortality in mice with myocardial infarction. *J Am Coll Cardiol* 2003 **41**: 2084-2092.

Whittington HJ, Babu GG, Mocanu MM, Yellon DM, and Hausenloy DJ. The diabetic heart: Too sweet for its own good? *Cardiol Res Pract* 2012 **2012**: 845698.

Yellon DM, and Hausenloy DJ. Myocardial reperfusion injury. *N Engl J Med* 2007 **357**: 1121-1135.

Table 4.1 Systemic and cardiac morphologic characteristics of T1D male and female mice.

	Male		Female	
	Ctrl	STZ	Ctrl	STZ
Blood glucose (mM)	8.1 ± 0.4	23.0 ± 2.1*	8.2 ± 0.3	13.4 ± 1.6* [#]
Body weight (g)	31.3 ± 1.8	25.8 ± 0.6*	26.4 ± 1.3 [#]	22.8 ± 0.5*
Tibia length (mm)	17.1 ± 0.1	16.7 ± 0.3	17.7 ± 0.7	16.8 ± 0.2
Heart weight (mg)	167 ± 6	143 ± 8*	145 ± 8 [#]	126 ± 5
Heart weight/body weight (mg/g)	5.5 ± 0.3	5.6 ± 0.3	5.2 ± 0.2	5.5 ± 0.1
Heart weight/tibia length (mg/mm)	9.7 ± 0.3	8.6 ± 0.5	7.9 ± 0.4 [#]	7.4 ± 0.3

Data are presented as mean ± sem (n=8-13/grp). *p<0.05 vs. Ctrl, [#]p<0.05 vs. male, 2-way ANOVA, annotated with LSD post-hoc analyses. Heart weights were measured post ischemia-reperfusion.

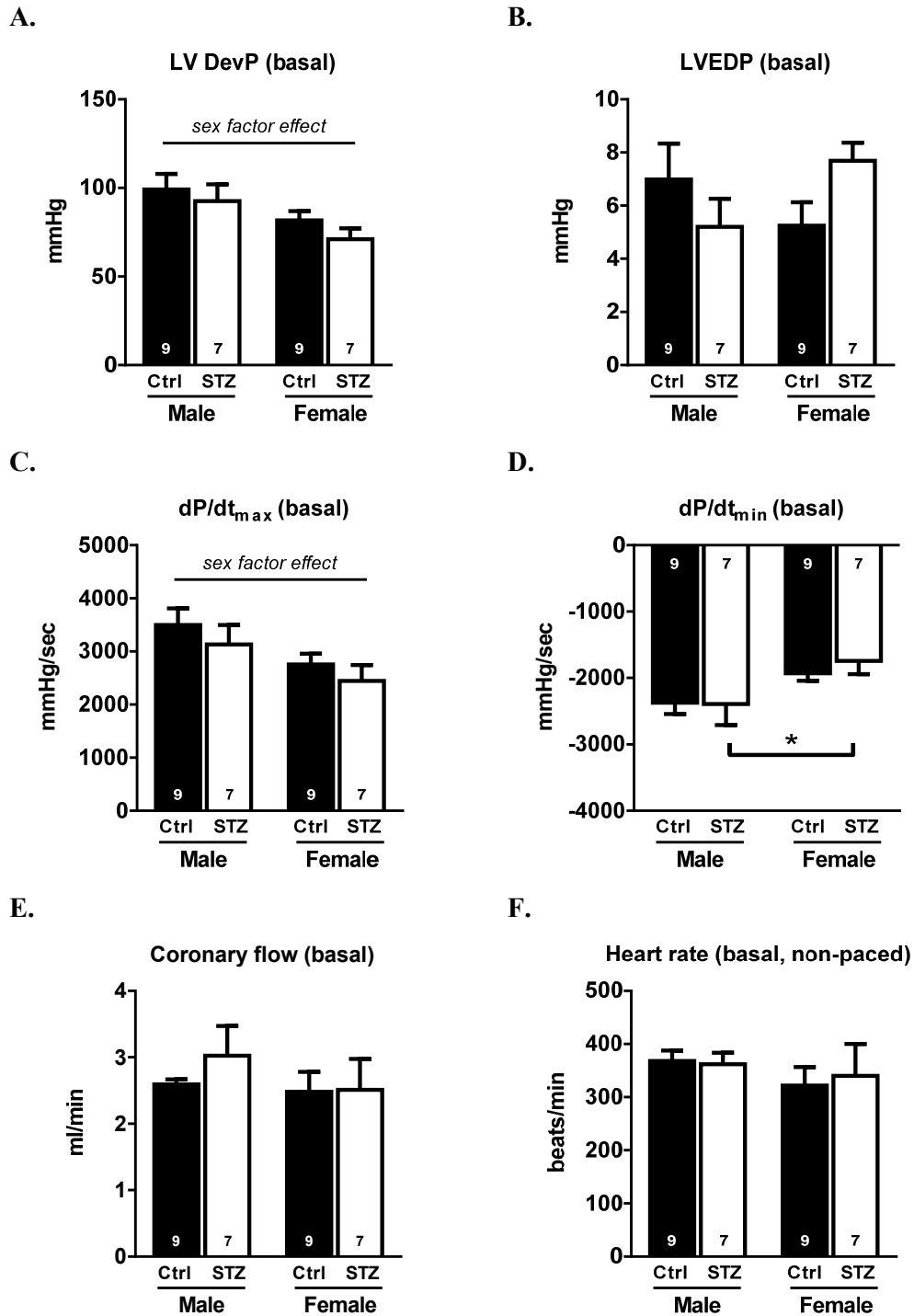


Figure 4.1. No effect of T1D on basal left ventricular function in *ex vivo* male and female mouse hearts. Post 15 mins perfusion equilibration period, hearts were paced at 420 beats per min. **A.** Left ventricular developed pressure (LV DevP). **B.** Left ventricular end-diastolic pressure (LVEDP). **C.** Left ventricular maximum rate of contraction (dP/dt_{max}). **D.** Left ventricular maximum rate of relaxation (dP/dt_{min}). **E.** Coronary flow rate. **F.** Heart rate, pre-pacing. Data are presented as mean $\pm$ sem. *p<0.05, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

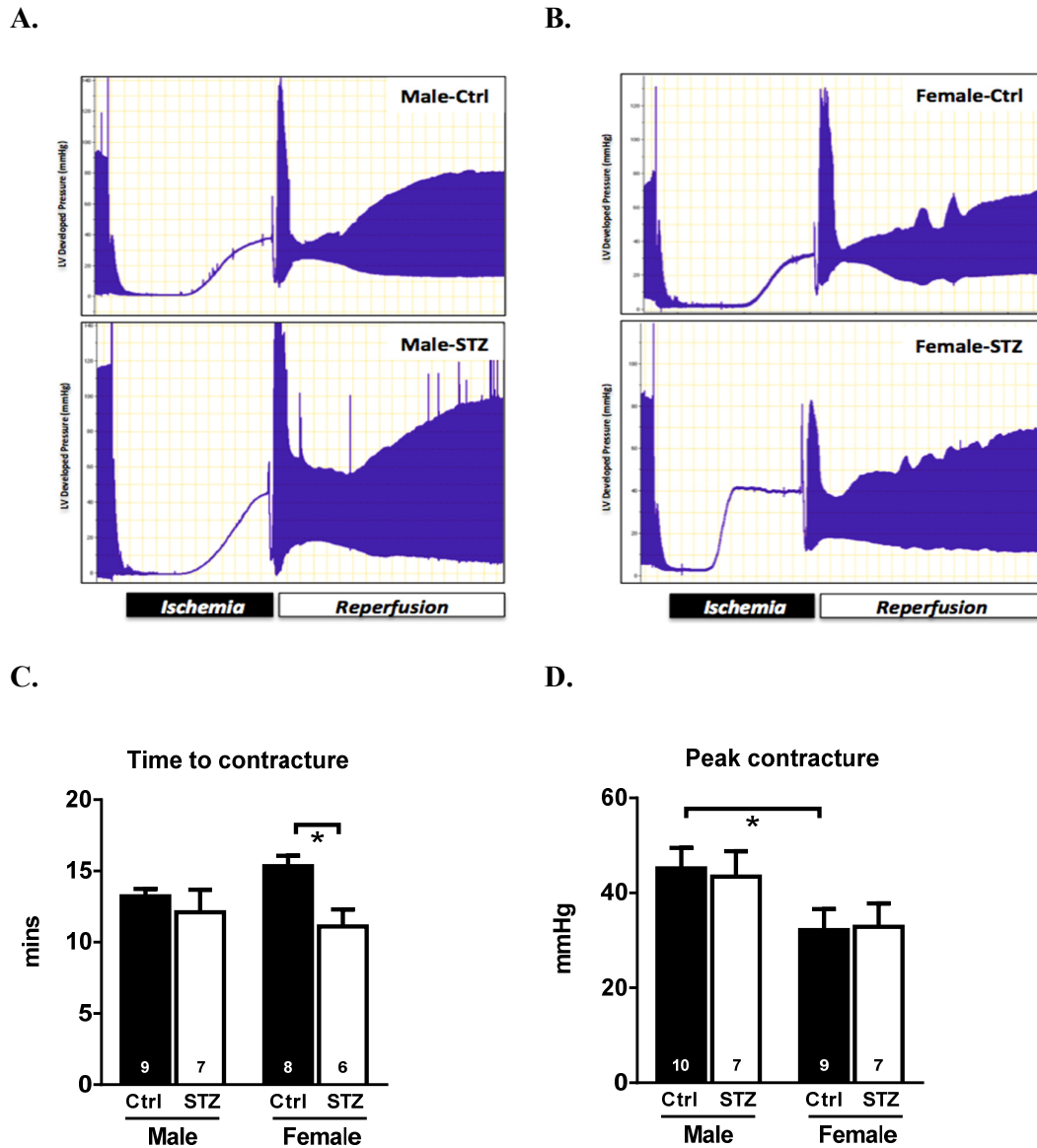


Figure 4.2. Sex differences in ischemic injury in T1D mice. **A.** Representative pressure traces of male control and STZ mouse hearts throughout the ischemia-reperfusion protocol. **B.** Representative pressure traces of female control and STZ mouse hearts throughout the ischemia-reperfusion protocol. **C.** Time to onset of ischemic contracture. **D.** Amplitude of ischemic contracture. Data are presented as mean $\pm$ sem. * $p < 0.05$, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

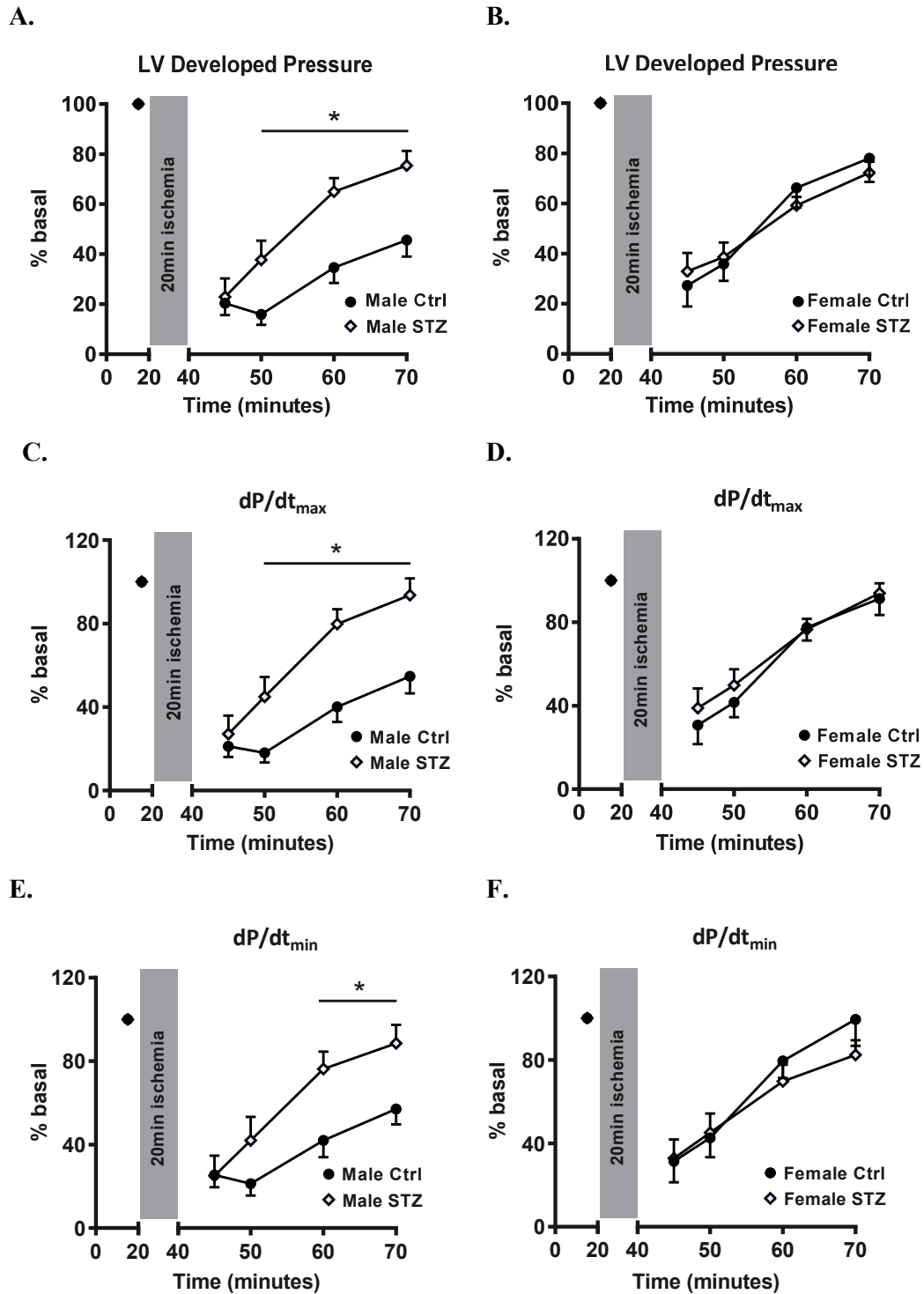


Figure 4.3. Sex differences in the recovery of contractile function post 20min ischemia in T1D mice. A, B. Percentage recovery of systolic function (left ventricular developed pressure, LV DevP). C, D. Percentage recovery of left ventricular maximum rate of contraction (dP/dt_{max}). E, F. Percentage recovery of left ventricular maximum rate of relaxation (dP/dt_{min}). Data are presented as mean $\pm$ sem. * $p < 0.05$, Students' t-test between Ctrl and STZ groups at each timepoint.

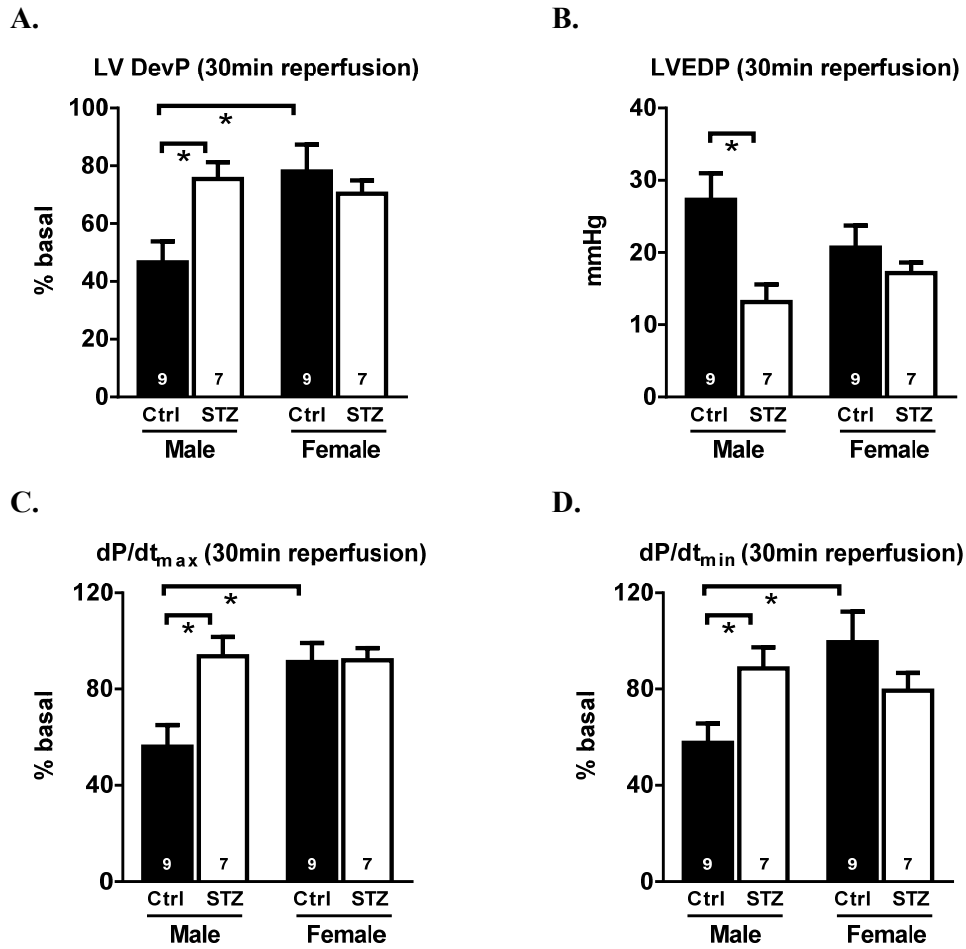


Figure 4.4. Left ventricular function at 30 mins reperfusion of *ex vivo* T1D male and female mice hearts. **A.** Percentage recovery of systolic function (left ventricular developed pressure, LV DevP). **B.** Left ventricular end-diastolic pressure (LVEDP). **C.** Percentage recovery of left ventricular maximum rate of contraction (dP/dt_{max}). **D.** Percentage recovery of left ventricular maximum rate of relaxation (dP/dt_{min}). Data are presented as mean $\pm$ sem. * $p < 0.05$, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

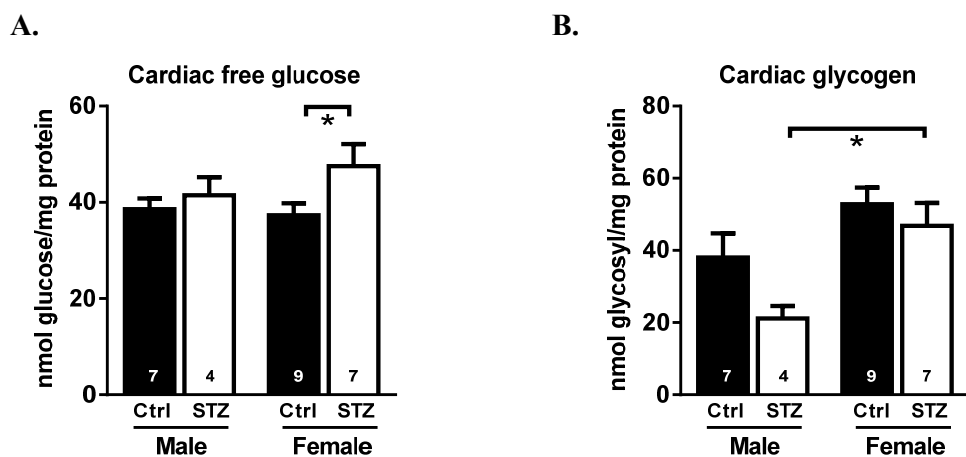


Figure 4.5. Differential glycogen utilisation in male and female T1D mouse hearts post ischemia-reperfusion. A. Cardiac free glucose content normalised to protein. **B.** Cardiac glycogen content normalised to protein. Data are presented as mean $\pm$ sem. * $p < 0.05$, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

CHAPTER 5

A signalling pathway survey in type 1 and type 2 diabetic myocardium of female and male mice

Manuscript generated from this chapter:

Chandramouli C, Reichelt ME, Ma, B, Qin CX, De Blasio M, Ritchie RH, Mellor KM[#], Delbridge LMD[#]. Distinctive cardiomyocyte trophic, viability and energy regulatory signalling pathways in type 1 and type 2 diabetic myocardium of female and male mice. *Am J Physiol Heart Circ Physiol*. [In preparation].

5.1 Introduction

Findings from the preceding two chapters demonstrated that female diabetic cardiac structural and functional pathologies were more marked despite modest changes at the systemic level. This sex disparity may be attributable to diminished cardiac glycogen utilisation and a differential diabetic glucose handling response in females. This chapter provides an operational snapshot of gene expression changes in male and female T1D myocardium to further elucidate metabolic adaptation in diabetes. Beyond the well-defined T1D and the classical obesity-related T2D, there is renewed interest in the underweight or normal weight lean ($25 < \text{BMI} < 18$) diabetes that is so far not well characterised (George *et al.*, 2015). Therefore, this chapter also compares the T1D findings to those from T2D models (lean and obese) and investigates early metabolic and energy stress responses in diabetes between cardiac and skeletal muscle. The goal of this chapter is to compare and contrast the differences in gene profiles between different diabetic states and tissues.

Alterations in myocardial substrate metabolism and energetics in diabetes is an important early adaptive response in the heart (Han *et al.*, 2014; Liu *et al.*, 2001; Pham *et al.*, 2014; van Heerebeek *et al.*, 2008). Suppression of P13K/Akt insulin signalling in insulin-deficient or insulin resistant settings is linked with reduced GLUT4 glucose transporters on the plasma membrane and restricted glucose uptake (Boudina *et al.*, 2006; Desrois *et al.*, 2004). A decline in glycolysis and pyruvate oxidation is also observed in human diabetic patients (Peterson *et al.*, 2015) and animal models of diabetes (Boudina *et al.*, 2006) along with a shift to higher reliance on fatty acid oxidation for energy supply (An *et al.*, 2006). A paradoxical increase in glycogen has been documented in clinical (Mowry *et al.*, 1951; Sakakibara *et al.*, 2011; Warren, 1930) and experimental diabetic hearts (Alfarano *et al.*, 2011; Lajoie *et al.*, 2004; Shearer *et al.*, 2011). Rodent models have shown increases in cardiac glycogen content in parallel with a depletion of liver and skeletal muscle glycogen stores (Kokubun *et al.*, 2009). In addition, conflicting effects of fasting, adrenergic stimulation, hypoxia, insulin, and exercise on skeletal vs. cardiac muscle glycogen supplies were identified in a number of early pioneering studies (Dudley *et al.*, 1923; Evans, 1934). Thus, the occurrence of myocardial metabolic dysregulation in T1D is evident and tissue specificity is apparent, yet the fundamental basis for the differential metabolic responses in distinct muscle types is still not understood.

Clinical evidence of sex differences in glucose handling has been demonstrated in non-diabetic and T2D settings. A lower utilisation of glucose was associated with ageing female, but not male, hearts in healthy human subjects (Kakinuma *et al.*, 2013). With the onset of insulin resistance, PET-generated time activity curves reveal more marked reduction in glycolysis, glycogen deposition and glucose oxidation with T2D in men compared to women (Peterson *et al.*, 2015). Experimental studies have demonstrated a pivotal role for the estrogen receptor alpha (ER α) in maintaining sufficient myocardial glucose uptake in female mice (Arias-Loza *et al.*, 2012) and a direct regulatory effect of estrogen on the insulin pathway via upregulation of Akt (Patten *et al.*, 2004). This pathway plays a crucial role in the metabolic stress response via regulation of glucose transport and storage as glycogen. However, molecular mechanisms associated with this sex-specific metabolite handling response in a diabetic experimental setting have not been explored.

Intracellular glucose deprivation and impaired glucose signalling in the diabetic heart pose a significant increase in energy stress, which in turn causes aberration of cardiac macroautophagy (bulk protein degradation) (Mellor *et al.*, 2011) and glycogen-specific autophagy-“glycophagy” (Reichelt *et al.*, 2013). Interestingly, P13K/insulin and AMPK signalling are common regulators for glycogen handling and autophagy. Insulin signalling promotes glycogen storage via Akt inhibition of glycogen synthase kinase 3 β (GSK3 β) and suppresses autophagy via mammalian target of rapamycin (mTOR) inhibition of unc51-like kinase 1/2 (ULK1/2) (Jung *et al.*, 2010; Mammucari *et al.*, 2008). AMP-activated kinase (AMPK) indirectly promotes glycogen storage by increasing glucose uptake (Hunter *et al.*, 2011) leading to allosteric activation of glycogen synthase via glucose-6-phosphate. Unlike insulin signalling, AMPK promotes autophagy via activation of ULK1/2 (Mack *et al.*, 2012). Following the first evidence from Mellor *et al.* (2011) on increased cardiac autophagy in diabetic hearts, a number of experimental studies have shown increased (Lee *et al.*, 2012; Wang *et al.*, 2014), decrease (Xie *et al.*, 2011b; Xu *et al.*, 2015; Zhao *et al.*, 2013) or unchanged (Lee *et al.*, 2014; Marsh *et al.*, 2013) autophagic responses using various T1D and T2D models. The reason for this variability is neither attributable to different diabetic models nor the severity of diabetes (Delbridge *et al.*, 2015), as contrasting outcomes within the same model (STZ mouse, 150 mg/kg x1 injection) have been reported (Eguchi *et al.*, 2012; Xu *et al.*, 2013b). A proposed explanation is that the autophagic response is a net

regulatory outcome between AMPK and Akt signalling (Delbridge *et al.*, 2015) but this is yet to be confirmed, as changes in these key regulators of autophagy have not always been reported along with the hallmark autophagic markers. Therefore, the present study has for the first time conducted a more extensive evaluation of the autophagy machinery (in addition to the key autophagy markers) in different diabetic models in order to understand important differences in autophagy in both T1D and T2D.

Some evidence of sex differences in autophagy is evident from studies in non-diabetic settings. An increased susceptibility to macrophagy (increased LC3BII:I ratio) in female compared to male rat hearts when subjected to 30mins coronary artery occlusion has been reported. No accompanying changes in the apoptotic markers, Bax to Bcl2 ratio have been reported in females, which is in contrast to the marked rise in males (Chen *et al.*, 2013). A more pronounced activation of autophagic markers in females has also been reported in other non-cardiac cells (Maselli *et al.*, 2009; Straface *et al.*, 2009). An increase in glycophagic response observed in females following a 48 hour nutrient-deprived stress model is absent in males. This is associated with selective increase in activation of AMPK, Akt, glycogen tagging protein, STBD1 and glycophasosomal protein, GABARAPL1, in females (Reichelt *et al.*, 2013). Sex differences in diabetic macroautophagic and glycophagic responses in diabetic hearts are yet to be delineated.

Cardiac-specific metabolic alterations in diabetes are evident and have been associated with cell death pathology. In addition to apoptotic cell loss, evidence of phagic processing of macromolecules and glycogen in diabetic hearts has emerged in recent years. The utilisation of various experimental diabetic rodent models with diverse metabolic perturbation profiles has made it difficult to reach a consensus. Sex-specific differences have not been explored. Therefore, this study examines the fundamental molecular changes in glycogen handling, glycophagy, autophagic, insulin and AMPK signalling in diabetic hearts. First, sex differences are examined using the STZ-induced T1D mouse model utilised in this thesis so far. In addition, changes in a genetic and a dietary T2D model are compared against findings from a T1D setting. Finally, comparative tissue analysis in an acute T1D setting is performed using cardiac and skeletal muscle. Novel findings reported in this chapter include sex-specific glycogen handling in T1D, with increased enzyme-regulated glycogen breakdown in females and increased “glycophagic”- bulk breakdown in males with T1D. A pronounced increase in autophagic drive was also evident in female but, not male T1D. In addition, both lean

and obese T2D models depicted sharp contrasts in metabolic and autophagic gene expression. In an acute insulin withdrawal T1D setting, cardiac and skeletal responses were not different from one another.

5.2 Specific Methodology

All animals utilised in this study were handled in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (NHMRC/CSIRO/ACC, 2013). All the procedures involved were approved by Animal Ethics Committee of the University of Melbourne (AEC Approval Numbers: 1112184, 1101838, 1101784).

5.2A Animal models of diabetes

Four different animal models of diabetes were used in this study. Experiments pertaining to Dietary lean T2D (Fructose-fed) and Genetic Obese T2D (db/db), with the exception of molecular work detailed in chapter, were performed by other lab groups as described below. The systemic characteristics of the different models are given in Table 5.1.

Chronic T1D—Male and female mice on C57Bl/6J background (n=3/group) were utilised for chronic STZ induction. As for Chapter 3 and 4, diabetes was induced at 15 weeks of age with 5 daily injections of Streptozotocin (STZ, 55 mg/kg; Sigma-Aldrich MO, USA) as described previously and detailed in Section 2.4. The mice were followed for a further 8 weeks, with tissue collection at 23 weeks of age.

Acute T1D—Male mice on C57Bl/6J background (n=3/group) were used to assess the early response to insulin withdrawal in an acute setting as described in Section 2.2. Five daily STZ injections (55 mg/kg of STZ; Sigma-Aldrich, MO, USA) were administered at 7 weeks of age. Blood glucose and body weight were monitored until the average blood glucose increased by 50% from baseline (~2 days post STZ injections).

Dietary lean T2D—Cardiac tissues from fructose-fed mice were a kind gift from Dr. Kimberley Mellor, Department of Physiology, University of Auckland. Male C57BL/6 mice aged 4 weeks were fed with either a control (55% starch; AIN93G) or high fructose diet (60% fructose; SF03-018) (Speciality Feeds, WA, Australia) for 12 weeks. These mice exhibited glucose intolerance, approximately 10% increase in blood glucose levels and no change in plasma insulin, body weight or cardiac weight index (Table

5.1). Tissues were collected upon completion of the dietary intervention at 16 weeks of age.

Genetic obese T2D – RNA extracted from 10-week-old spontaneously diabetic male C57BL/6 *db/+* and *db/db* mice (n=3/group) was a generous gift from Assoc.Prof. Rebecca Ritchie, Baker IDI Heart and Diabetes Institute, Melbourne, Australia. *db/db* mice exhibit marked increase in blood glucose, body weight and plasma insulin and a reduced cardiac weight index (Table 5.1).

5.2B Tissue harvesting

Upon completion of the respective interventions, mice were anaesthetised using sodium pentobarbitone (100mg/kg, i/p.). Tissue collection was performed as previously described in Section 2.8. Ventricular tissues and quadriceps femoris were blotted on filter paper, weighed on an analytical balance and snap-frozen in liquid N₂ to be stored at -80°C until further analysis.

5.2C RNA extraction and reverse transcription

RNA was extracted from snap-frozen ventricular tissues by homogenisation in TRIzol® followed by purification using the PureLink RNA mini kit (Invitrogen, CA, USA) as described in Section 2.15. Reverse-transcription of purified RNA into cDNA was performed using the RT² First Strand Kit (Qiagen, CA, USA) as described in Section 2.16.

5.2D RT profiling gene expression analysis

Plates with 64 genes x 6 sample customised mouse RT² PCR Array (Qiagen, CA, USA) were used to examine gene expression analysis on a Biorad CFX384 (Biorad, CA, USA) thermocycler as detailed in Section 2.16. The selected genes included 59 genes of interest, 2 reference genes (18S and ACTB), a genomic DNA control, a reverse transcription control (RTC) and a positive PCR control (PPC). A list of genes of interest, gene symbols and accession numbers is provided in Table 2.1 in Chapter 2. Thermal cycling conditions used for real-time PCR were 1 min at 95°C, 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C each. Exported Ct values were analysed using the comparative $\Delta\Delta C_t$ method.

5.3 Results

5.3A Metabolic and cell death gene expression contrast in male and female T1D

To determine whether sex differences in diabetic cardiopathology resulted from corresponding gene expression changes in energy utilisation and cell death pathways, the steady state mRNA abundance of the respective genes was quantified using RT² Profiler PCR Custom Arrays. Stylised heat maps illustrating the general trends observed in genes from glycogen handling, glycolysis, macroautophagy, autophagy machinery, apoptosis, signalling and metabolic pathways were generated based on fold change in diabetic group (vs. respective controls) and statistical significance (Figure 5.1A). Genes which are significantly upregulated ($p < 0.05$) and display a trend in upregulation ($0.05 < p < 0.1$) and are coded as bright red and pale red respectively. Genes that are significantly downregulated ($p < 0.05$) and display a trend in downregulation ($0.05 < p < 0.1$) are coded as bright blue and pale blue respectively. Gene expression changes which were significant and cellular pathways which showed tendencies of up/downregulation in the STZ group in both sexes are discussed below.

From the 57 genes examined, 2 genes were significantly upregulated with STZ and 8 genes were significantly downregulated with STZ in male mouse hearts. In contrast, 15 genes were upregulated and 1 gene was downregulated in STZ female mouse hearts (Figure 5.1B). Expression of genes involved in insulin and AMPK signalling pathways was not markedly different in T1D in both sexes, with exception of a few insulin signalling regulatory genes (male, *Akt 1*, 0.84-fold; female, *Pik3r4*, 1.21-fold). In males, 4 out of 8 genes from glucose metabolic pathway were downregulated in response to chronic STZ. Interestingly, the only common STZ-induced gene expression change in males and females was a significant decrease in *Glucokinase* (male, 0.75-fold; female, 0.53-fold), suggesting that downregulation of glucose phosphorylation is similar in T1D males and females. An opposing directional STZ-induced change in gene expression between the sexes was evident in the key regulator of glycolytic pathway, *Phosphofructokinase* (male, 0.75-fold; female, 1.16-fold). *Phosphoglucomutase 2* (1.19-fold) was higher in expression in female but not male STZ.

The lack of STZ-induced differences in macrophagy genes in female mouse hearts was surprising considering a distinct pattern of upregulation of 8 out of 20 genes involved in autophagic machinery (*Pi3kc3*, *Atg16l1*, *Atg4b*, *Atg4c*, *Atg12*, *Lamp2*, *Gabarap*,

Dram2) in female STZ but not male STZ mice. Some STZ-induced differences in lysosomal protease genes were present in males but not females (*Cathepsin B*, 0.78-fold and *Cathepsin*, 1.27-fold). This female-specific upregulation in autophagy machinery genes was in contrast to downregulation observed in males with STZ (*Atg7*, 0.46-fold). STZ downregulated genes were involved in glycogen synthesis (*Synthase 1*, 0.6-fold) and upregulated genes involved in glycophagy-mediated glycogen breakdown (*Gabarapl1*, 1.5-fold) in males but not females. In contrast, STZ upregulated genes were involved in cytosolic glycogen breakdown in females but not males (*Agl*, 1.5-fold; *Phosphorylase*, 1.3-fold). There were no major STZ-induced differences observed in hallmark apoptotic genes (*Bad*, *Bcl2*, *Bnip3*, *Dapk1*) in males or females, with the exception of one apoptotic gene upregulated in females but not males (*Caspase 3*, 1.49-fold). Hence, it appears that STZ males may have more capacity to access glycogen stores via glycophagy, whereas females resource their glycogen utilisation via cytosolic breakdown. Although the first step of glycolysis (*Glucokinase*) is downregulated with STZ in both sexes, males exhibit decreased glycolytic pathway genes while females have some evidence of upregulation in glycolytic drive. In addition, upregulation in autophagic machinery genes was evident in females with STZ, but not in males.

5.3B Gene expression profile differs between T1D and T2D, and lean and obese diabetic models

The diversity of experimental models in T1D and T2D has made understanding of diabetic cardiopathology particularly challenging. Animal models recapitulate varying extents of the diabetic phenotype similar to variability observed in the clinical setting, often linked with other co-morbidities. Evaluation of gene expression profiles utilising different diabetic models can be more informative. To determine if the metabolic and cell death signalling changes observed in T1D models extend to other diabetic experimental models, gene profiling was performed in fructose-fed lean T2D mouse and a *db/db* obese T2D mouse models as shown in Figure 5.2A. The STZ male mouse heat map from Figure 5.1A is presented again in Figure 5.2A for visual comparison between T1D and T2D. Systemic characteristics of individual models have been summarised in Table 5.1 based on previous published data from these mice (Bowden *et al.*, 2015; Mellor *et al.*, 2011). A varying degree of hyperglycaemia was present in all models, with STZ females and fructose-fed males showing less pronounced increases than the other groups. Body weight was reduced in STZ males and fructose-fed males as

opposed to increased body weight observed in *db/db* mice and no difference in STZ females. Glucose intolerance was consistent in all models. Plasma insulin levels were diminished in STZ males and females (to a lesser extent) while unchanged in fructose-fed and increased in *db/db* mice (Table 5.1). The varying obesity and insulin status may have significant impact on gene expression changes in intracellular signalling and energy metabolism.

Expression of genes from P13K/Akt signalling cascade was evidently increased with diabetes in lean T2D but not in obese T2D (*Akt*, 1.62-fold; *Ulk1*, 1.91-fold). Despite no differences in Akt signalling genes in obese T2D, decreased expression of downstream autophagic modulators was evident (*Ulk1*, 0.49-fold), possibly driven by reduced AMPK signalling (*AMPK*, 0.58-fold). In contrast to the downregulation in glucose metabolism observed in T1D, there was indication of increased glucose metabolism in lean T2D but not obese T2D (*Hexokinase 2*, 1.88-fold).

Diabetes-induced increased expression of autophagic machinery genes was observed in lean T2D but not obese T2D (*Pik3c3*, *Atg16l2*, *Atg3*, *Atg4b*, *Atg9a*, *Atg12*, *Ambra1*, *Lamp1*). In contrast, obese T2D depicted pronounced diabetes-induced downregulation of autophagy machinery genes (*Pik3c3*, *Atg16l1*, *Atg16l2*, *Atg4b*, *Atg5*, *Atg9a*, *Atg12*) while T1D did not show any difference. With diabetes, key autophagic modulators were upregulated in lean T2D (*Gabarap*, 2.2-fold; *Dram2*, 2.25-fold) and downregulated in obese T2D (*Pink 1*, 0.70-fold; *Dram2*, 0.56-fold). There was an indication of reduced autophagy in obese T2D (*p62*, 0.68-fold) but not in lean T1D or lean T2D. Lower expressions of lysosomal proteases were evidenced in T1D and lean T2D (T1D, *Cathepsin B*, 0.78-fold; lean T2D, *Cathepsin D*, 0.78-fold). Interestingly, genes involved in glycophagic breakdown of glycogen were upregulated with diabetes in lean T1D and lean T2D (*Gabarapl1*, lean T1D, 1.47-fold; lean T2D, 2.73-fold) and downregulated with obese T2D (*Gabarapl1*, 0.44-fold; *STBD1*, 0.77-fold). Diabetes-induced changes in apoptotic genes were evident, with downregulation evident in both lean (*Bcl2*, 0.45-fold) and obese T2D (*Bnip3*, 0.63-fold). Collectively, glycogen utilisation and cell death pathways are upregulated in lean T2D and downregulated in obese T2D. The little overlap among the three models may be attributable to the varying insulin status and degree of hemodynamic loading on the heart in these different rodent models of diabetes.

5.3C Cardiac muscle fuel response is not different from skeletal muscle

To determine early diabetic metabolic and cell death responses between cardiac and skeletal (i.e. quadriceps femoris) muscle tissue, gene expression analysis was carried out on both tissues following acute insulin withdrawal (Figure 5.3A). Pronounced acute T1D-induced increases in glycogen handling genes were evident in both tissues (quadriceps, *Gsk3a*, 2.53-fold increase; cardiac, *GP kinase γ 1*, 1.79-fold). Interestingly, a glycophagy marker was upregulated in cardiac but not quadriceps muscle (*Gabarapl1*, 1.60-fold) and this upregulation was sustained throughout from an early acute insulin withdrawal stage up to 8 weeks of chronic T1D (compare to Figure 5.1A). Key macrophagy markers were not different with STZ in either tissue with the exception of downregulation in a lysosomal protease in quadriceps but not cardiac muscle (*Cathepsin B*, 0.77-fold). Some evidence of increased autophagic machinery was present in cardiac (*Pik3c3*, *Atg12*, *Atg 16|2*, *Gabarapl2*) and quadriceps femoris (*Atg9a*, 1.36-fold; *Atg 16|2* *Pink1*, 1.59-fold) muscles in this acute insulin withdrawal setting. In addition, STZ-induced increase in apoptotic cell death pathway was observed in both tissues (cardiac, *Caspase 3*, 1.49-fold; quadriceps, *Bcl2*, 1.79-fold) Interestingly, this early upregulation in *Caspase 3* in cardiac muscle was no longer different in the chronic T1D setting (compare to Figure 5.1A). Some evidence of downregulation of insulin signalling (*Pik2cg*, 0.77-fold) and glucose metabolism (*glucokinase*, 0.5-fold) was observed in the acute STZ heart, but not quadriceps; and downregulation of mTOR signalling was event in the quadriceps (*Mtor*, 0.81-fold), but not the heart. Notably, the early increase in cardiac autophagic machinery and apoptotic drive in acute insulin withdrawal setting was lost in the chronic T1D setting but increased glycophagic genes were sustained throughout. Although some instances of acute T1D-induced gene expression changes were tissue-specific, the majority of gene expression changes were in the same direction in cardiac and skeletal muscle, albeit statistical significance was not always attained in both tissue types.

5.4 Discussion

The primary goal of this chapter was to compare and contrast gene expression changes in key molecular pathways implicated with energy stress in diabetes. No study to date has compared gene expression profiles between different diabetic states and tissues. This present study has provided the first direct comparison of metabolic gene responses to diabetes in T1D males/females, lean/obese T2D and cardiac/skeletal muscle. The novel finding that female-specific increased cardiac autophagic drive is evident in T1D provides new evidence to support the contention of an underlying sex-specific macrophagic signalling disturbance in T1D. Sharp contrasts in diabetes-induced genomic changes between lean and obese T2D models were also evident. A net upregulation in phagic and metabolic genes was observed in the lean T2D mouse heart, while suppression was seen in the obese T2D mouse heart. Gene responses in the acute diabetic setting were comparable between cardiac and quadriceps femoris muscle. This new screening data provides new molecular leads for future investigations examining therapeutic targets for both T1D and T2D. These findings suggest that diabetic molecular pathology is distinctly different between the sexes, between diabetic states and is dependent on the degree of hemodynamic loading in the heart.

5.4A Insulin signalling is different in various diabetic settings

In the present study, diminished insulin and phosphoinositide 3-kinase (PI3K) signalling in chronic but not acute T1D males was consistent with reports from other insulin-deficient studies (Ceylan-Isik *et al.*, 2006; Huisamen, 2003). The lack of differences in female T1D could be attributed to estrogen modulation of insulin signalling pathway as previously demonstrated in various studies (Bell 2008; Patten 2004; Murphy 2011; Camper-Kirby 2001). One other study from the literature examining sex differences in acute T1D (2 weeks of STZ) reported no changes in total Akt protein expression in males. Interestingly, sex differences emerged in post translation regulation of Akt, with increased phosphorylated Akt in males, while it remained unchanged in females (Ceylan-Isik *et al.*, 2006). Determination of protein level (of selected genes) is an important future step in these studies, and the RT profiling data provides leads for these investigations. Indeed, directional changes in gene expression and protein level regulation of Akt have at times been reversed. Increased *Akt* gene expression in lean T2D was linked with previous demonstration of

reduced p-Akt/Akt protein expression and reduced insulin signalling in the same model (Mellor *et al.*, 2011).

5.4B Dysregulation in AMPK with varying intracellular energy levels

Dysregulation in AMPK, an energy-sensing enzyme that is activated when cellular energy levels are low, was evident in various diabetic settings. *AMPK* expression was significantly reduced in obese T2D, similar to previous observations using the *db/db* model (Kanamori *et al.*, 2015). A possible explanation for the differences in AMPK expression was high intracellular energy availability in obese models preventing an energy deprivation-induced increase in the AMP/ATP ratio which in turn increases *AMPK* (Kanamori *et al.*, 2015). A pronounced decrease in glycolysis was evident in T1D as observed in various studies (Da Silva *et al.*, 2012; Malfitano *et al.*, 2015; Mansor *et al.*, 2013). In female T1D, increased glycolytic drive was evidenced despite the trend towards a reduction in insulin levels (Figure 3.1B). Albeit not investigated in the present study, reduced GLUT transporters on the sarcolemmal membrane and glucose uptake are commonly observed in insulin-deficient-T1D males (Camps *et al.*, 1992) and in a sex-pooled study (Myers *et al.*, 2016). A human T2D study has reported comparable sex differences in glycolytic rate to be decreased in men and unchanged in women observed through positron emission tomography (Peterson *et al.*, 2015). Therefore, there was some evidence of gene-level reduction in insulin signalling and glycolysis in chronic T1D males, which were absent in an acute setting and in females. Lean and obese T2D mice displayed higher and lower AMPK signalling respectively accompanied by less marked changes in glucose metabolism, compared to T1D. Collectively, these findings suggest that AMPK may be an important nexus point of energy-sensing which may determine differential metabolic outcomes in different diabetic models.

5.4C Preferential glycogen processing routes in diabetic males and females

Myocardial glycogen exists in cytosol as “free” glycogen or is encapsulated in vacuolar autophagosomal bodies (Kotoulas *et al.*, 2006). Cytosolic glycogen breakdown is tightly regulated by glycogen phosphorylase and glycogen debranching enzyme actions. Phagic processing of glycogen involves STBD1 tagging of glycogen for lysosomal bulk degradation and binding to GABARAPL1 on phagosomal membranes (Jiang *et al.*, 2010). Interestingly, the cardiac glycopphagic marker, *Gabarapl1*, was significantly

altered with diabetes in all male models studied, regardless of the type of diabetes. The upregulation observed in chronic T1D males and lean T2D was comparable to increased GABARAPL1 protein expression following 48 hours non-pathological nutrient stress in male and female mice (Reichelt *et al.*, 2013).

Gabarapl1 is transcriptionally regulated by estrogen (Pellerin *et al.*, 1993) and was increased to a higher extent in females (compared to the increase in males) following nutrient deprivation (Reichelt *et al.*, 2013). Together, this led us to hypothesise an exacerbated increase in T1D females. Surprisingly, no difference in glycophagic breakdown was observed but an increase in enzyme regulated-cytosolic breakdown of glycogen was observed in T1D females. This suggested possible disruption in the phagic glycogen breakdown process and an increase in conventional glycogen breakdown in females under a chronic (8 weeks STZ) insulin deficient state. Current evidence of impaired phagic processing available from Pompe disease, caused by lysosomal acid alpha glucosidase deficiency, delineate severe impacts on cardiac muscle (Raben *et al.*, 2009). Although a concomitant diabetes-induced increase in glycogen was not evident at a basal setting in the present study (Figure 3.7C), reduced utilisation of glycogen under ischemic stress in females (Figure 4.5B) associated with sooner onset of ischemic contracture was apparent (Figure 4.2A).

A higher reliance on fatty acids as energy substrate could explain the downregulation in most of the glucose and glycogen handling genes observed in the obese T2D model. Indeed, in young (4 weeks) *db/db* mice, an increase in cardiac fatty acid oxidation precedes any changes in circulating substrate (Buchanan *et al.*, 2005), suggesting that shifts towards higher fatty acid utilisation may occur at early time points in genetic diabetic mice models. Interestingly, early increases in glycogen breakdown genes were observed in cardiac tissue but not in quadriceps, highlighting the importance of glycogen as a substrate fuel for the heart. Indeed, differences in cardiac and skeletal glycogen metabolism in various metabolic stress settings were established in early studies (Evans, 1934; Rabinowitz, 1971). Collectively, these preliminary gene profiling results point towards sex and tissue-specific glycogen processing routes, and further investigation of pathway activity is now warranted. Upregulation of key regulatory genes involved in bulk breakdown of glycogen was evident in acute T1D, chronic T1D and lean T2D male hearts while a less-efficient conventional breakdown was present in chronic T1D female hearts.

5.4D Evidence sex differences in macrophagic signalling in chronic T1D

This investigation is the first to provide evidence of sexual dimorphism in T1D macrophagy response in the heart. As reviewed in detail in Delbridge *et al.* 2015, diabetes is associated with increased (Mellor *et al.*, 2011; Russo *et al.*, 2012; Wang *et al.*, 2014), decreased (Pei *et al.*, 2015; Xie *et al.*, 2011a) or unchanged (Lee *et al.*, 2014; Marsh *et al.*, 2013) cardiac macrophagy in both T1D and T2D rodent models – but direct comparison of these models, within one experimental study, has not previously been reported. The general consensus is that macrophagy can be beneficial and pro-survival during acute stress but could have deleterious effects when chronically elevated (Kaye *et al.*, 2007; Nishida *et al.*, 2009; Palomeque *et al.*, 2009).

The hallmark genes involved in macrophagy LC3B and p62 were not different in most diabetic settings examined in this present study but post-translational modification at protein level is possible. Some evidence of reduced macrophagy is evident from other gene expression changes in chronic T1D males. The significant downregulation in *Cathepsin B* in chronic T1D males and *Atg7*, an E1-like enzyme responsible for lipidation of LC3 during phagophore closure, in both acute and chronic T1D males suggests possible downregulation in macrophagy, consistent with many T1D studies reporting downregulation in macrophagy with reduced LC3II/I ratio (He *et al.*, 2013; Xie *et al.*, 2011b; Zhao *et al.*, 2013) and Beclin protein expression (Zhao *et al.*, 2013). Conflicting increased cardiac autophagy in T1D males has also been reported (Kanamori *et al.*, 2015). These differences can be attributed to a higher dosage of STZ utilised (250 mg/kg STZ) and higher AMPK activity (Kanamori *et al.*, 2015). One other study reported an increase in macrophagy with 4 week STZ duration (Wang *et al.*, 2014), comparable to findings from acute T1D in both cardiac and quadriceps muscle in this study. The absence of both Akt and AMPK signalling data in Wang *et al.* (2014) makes it difficult to gauge the severity of signalling impairment preceding macrophagy and compare it against the present study.

In females, a pronounced increase in macrophagic drive (8 genes) presents, suggesting a female-specific modulation of macrophagy. This was surprising given that estrogen is shown to upregulate Akt signalling in female hearts (Bell *et al.*, 2008; Patten *et al.*, 2004), which in turn would result in mTOR-mediated suppression of macrophagy. Conversely, the increase in the macrophagic drive could be attributed to energy stress following a trend towards an increase in AMPK which relieves mTOR inhibition and

results in increased macrophagic signalling (Figure 5.1A), as depicted in glucose-starved cells (Kim *et al.*, 2011). While no previous study has directly compared male and female cardiac autophagy in diabetes, some insights can be gained from reports on autophagy in non-diabetic and non-cardiac tissues. A study examining sex differences in autophagy during ischemic stress has reported higher LC3BII/I ratios in female and lower ratios in male hearts subjected to 30 mins coronary artery occlusion (Chen *et al.*, 2013). Similarly, increased macrophagic genes (increased mRNA levels of *Atg4a*, *Atg16l1*, *Beclin 1* and *Ambra*) in female hearts in comparison to males were also observed in an avian model of enhanced growth rate (Piekarski *et al.*, 2014). Conflicting reduction in macrophagy has also been reported in female hearts in other pathologies such as colon cancer (Cosper *et al.*, 2011) and viral myocarditis (Koenig *et al.*, 2014). No sex differences were reported in macrophagy under nutrient stress (Reichelt *et al.*, 2013). One study with both sexes also reported diminished macrophagy following STZ administration but male and female data were pooled and no sex-specific comparisons were conducted (Xu *et al.*, 2013b). Therefore, this study provides novel evidence of a female-specific increase in macrophagic drive in T1D myocardium, uncovering potential for new sex-specific therapeutic targets to be explored.

5.4E Contrasting macrophagic response in lean and obese T2D

Increased macrophagy in lean T2D, was observed from elevation in LC3BII/I protein ratios reported by Mellor *et al.* (2013) in the same fructose-fed model. This was associated with suppressed P13/Akt signalling, increased reactive oxygen species formation and increased myocyte loss (Mellor *et al.*, 2011). Interestingly, macrophagic response in obese T2D was in contrast with lean T2D. The downregulation in autophagic machinery genes involved in formation of phagosomes was consistent with reduced autophagy observed in other obese T2D rodent models (Marsh *et al.*, 2013; Pei *et al.*, 2015). Conflicting findings from Munasinghe *et al.* (2015) concluded an increase in autophagy with a marked increase in *Beclin*, the gene involved in autophagy initiation, and *LC3B* expression (Munasinghe *et al.*, 2016) in obese T2D. Interestingly, autophagic machinery genes (*Atg 4A* and *Atg 3*) were downregulated in their study. Despite increases in LC3B and p62 expression, Kanamori *et al.* (2015) have evidenced reduced autophagy using the same obese T2D model. They ascribed these differences to immature autophagosomes, delayed autophagic flux and reduced lysosomal activity resulting in suppression of autophagy at the final digestion step ((Kanamori *et al.*,

2015). Similar inhibition of phagosome maturation was also observed in high-fat induced obese mice further emphasising the importance of autophagic flux and phagosome formation (Xu *et al.*, 2013a). Thus, macrophagic genes were upregulated in lean and downregulated in obese T2D, but further investigation into autophagic flux outcomes is now warranted.

5.4F Tendency of upregulated apoptotic signalling in female T1D

The interplay between apoptosis and autophagy has been shown to have an important role in diabetic cardiomyopathy associated with decreased cardiac muscle mass, interstitial fibrosis and impaired cardiac function (Cai *et al.*, 2002; Zou *et al.*, 2013). Increased *Caspase-3* gene expression in chronic T1D females which was absent in males was in line with higher cleaved Caspase-3 protein expression in females as early as 4 weeks after STZ-induced diabetes, while no significant changes were observed in males (Moore *et al.*, 2014). Interestingly, in the current study, the increase was significant in acute but not chronic T1D males. These findings were consistent with Moore *et al.* (2014) who reported no differences in total Caspase expression in males 8 weeks post STZ. However, the same study showed an increase in Cleaved-caspase-3 in male T1D levels, which was not measured in the present study. Increased Bcl2 to Bax ratios in males was absent in females following ischemia reperfusion injury in normoglycemic mice (Chen *et al.*, 2013). Some evidence of altered apoptotic gene expression in lean T2D and a possible downregulation in obese T2D was present, pending confirmation at protein level. In addition to macrophagy, apoptotic drive was also increased in females while remaining unchanged male chronic T1D.

5.4G Limitations

The acute T1D mouse model was generated to age-match with a different cohort of fasted animals for comparison of acute cardiac glycogen responses to metabolic stress. Given that the glycogen response was not as we expected, it was later decided to be used as a sex differences comparison in development of hyperglycemia. For future studies, age-matched acute T1D and chronic T1D mice could be considered.

5.5 Conclusions and next questions

The current chapter has built on the work from Chapter 3, which evidenced greater structural and functional deficits in female diabetic hearts, and Chapter 4, which demonstrated a female-specific substrate handling response. This chapter compared operational snapshots of male and female diabetes-induced alterations in key cell death and metabolic handling genes to explain the sexual dimorphism observed. The present study has provided novel evidence of sex differences in autophagy in the T1D setting. A female-specific increase in myocardial autophagic and apoptotic cell death was operational in chronic T1D in this study. Male chronic insulin deficiency in this study was associated with a greater tendency of glycophagic bulk breakdown of glycogen. The “quicker” and efficient access to glucose in diabetic hearts possibly alleviated the increase in energy stress causing no differences in cell death pathways and reasonably preserved cardiac function. In contrast, perhaps, the insulin-deficient female hearts exhibited increased autophagic drive to compensate for the inefficiency in glycogen breakdown. This excessive autophagy was also associated with increased apoptosis and significant structural and functional deficit in diabetes.

This chapter has also extended findings from the chronic T1D model to other rodent diabetic models of T2D. Contrasting responses in lean and obese T2D were found. Lean T2D hearts with insulin resistance displayed downregulated insulin signalling and increased autophagy. In addition to insulin resistance, the obesity phenotype in obese T2D resulted in a suppression of autophagy, possibly due to high intracellular nutrient energy status. Interestingly, cardiac autophagy has been previously reported to be increased in lean T2D mice (Mellor *et al.*, 2011) and decreased in obese T2D mice (Huynh *et al.*, 2012). This chapter intended to present an operational snapshot comparison between the primary focus of the thesis, the chronic T1D mice model, and T2D mice models.

Upregulation of the renin-angiotensin system is often observed in a diabetic setting (Lim *et al.*, 2004; Velloso *et al.*, 2006). Recent advances in autophagic studies have identified Angiotensin II (AngII) as a cardiac autophagy modulator (Porrello *et al.*, 2009a; Porrello *et al.*, 2009b). An estrogen response element localised to the angiotensinogen promoter may be instrumental in sex-specific regulation of local tissue

AngII production (Feldmer *et al.*, 1991; Mellor *et al.*, 2014). Given that the female diabetic phenotype has been demonstrated to be distinct with accentuated autophagic drive, the involvement of local cardiac renin-angiotensin system in exacerbation of diabetic functional pathology is evaluated in both sexes in the next chapter.

5.6 References

- Alfarano C, Suffredini S, Fantappie O, Mugelli A, Cerbai E, Manni ME, and Raimondi L.** The effect of losartan treatment on the response of diabetic cardiomyocytes to atp depletion. *Pharmacol Res* 2011 **63**: 225-232.
- An D, and Rodrigues B.** Role of changes in cardiac metabolism in development of diabetic cardiomyopathy. *Am J Physiol Heart Circ Physiol* 2006 **291**: H1489-1506.
- Arias-Loza PA, Kreissl MC, Kneitz S, Kaiser FR, Israel I, Hu K, Frantz S, Bayer B, Fritzemeier KH, Korach KS, and Pelzer T.** The estrogen receptor-alpha is required and sufficient to maintain physiological glucose uptake in the mouse heart. *Hypertension* 2012 **60**: 1070-1077.
- Bell JR, Porrello ER, Huggins CE, Harrap SB, and Delbridge LM.** The intrinsic resistance of female hearts to an ischemic insult is abrogated in primary cardiac hypertrophy. *Am J Physiol Heart Circ Physiol* 2008 **294**: H1514-1522.
- Boudina S, and Abel ED.** Mitochondrial uncoupling: A key contributor to reduced cardiac efficiency in diabetes. *Physiology (Bethesda)* 2006 **21**: 250-258.
- Bowden MA, Tesch GH, Julius TL, Rosli S, Love JE, and Ritchie RH.** Earlier onset of diabetes-induced adverse cardiac remodeling in female compared to male mice. *Obesity (Silver Spring)* 2015 **23**: 1166-1177.
- Buchanan J, Mazumder PK, Hu P, Chakrabarti G, Roberts MW, Yun UJ, Cooksey RC, Litwin SE, and Abel ED.** Reduced cardiac efficiency and altered substrate metabolism precedes the onset of hyperglycemia and contractile dysfunction in two mouse models of insulin resistance and obesity. *Endocrinology* 2005 **146**: 5341-5349.
- Cai L, Li W, Wang G, Guo L, Jiang Y, and Kang YJ.** Hyperglycemia-induced apoptosis in mouse myocardium: Mitochondrial cytochrome c-mediated caspase-3 activation pathway. *Diabetes* 2002 **51**: 1938-1948.
- Camps M, Castello A, Munoz P, Monfar M, Testar X, Palacin M, and Zorzano A.** Effect of diabetes and fasting on glut-4 (muscle/fat) glucose-transporter expression in insulin-sensitive tissues. Heterogeneous response in heart, red and white muscle. *Biochem J* 1992 **282 (Pt 3)**: 765-772.

Ceylan-Isik AF, LaCour KH, and Ren J. Gender disparity of streptozotocin-induced intrinsic contractile dysfunction in murine ventricular myocytes: Role of chronic activation of akt. *Clin Exp Pharmacol Physiol* 2006 **33**: 102-108.

Chen C, Hu LX, Dong T, Wang GQ, Wang LH, Zhou XP, Jiang Y, Murao K, Lu SQ, Chen JW, and Zhang GX. Apoptosis and autophagy contribute to gender difference in cardiac ischemia-reperfusion induced injury in rats. *Life Sci* 2013 **93**: 265-270.

Cosper PF, and Leinwand LA. Cancer causes cardiac atrophy and autophagy in a sexually dimorphic manner. *Cancer Res* 2011 **71**: 1710-1720.

Da Silva D, Ausina P, Alencar EM, Coelho WS, Zancan P, and Sola-Penna M. Metformin reverses hexokinase and phosphofructokinase downregulation and intracellular distribution in the heart of diabetic mice. *IUBMB Life* 2012 **64**: 766-774.

Delbridge LMD, Mellor KM, Taylor DJR, and Gottlieb RA. Myocardial autophagic energy stress responses—macroautophagy, mitophagy, and glycophagy. *American Journal of Physiology - Heart and Circulatory Physiology* 2015 **308**: H1194-H1204.

Desrois M, Sidell RJ, Gauguier D, Davey CL, Radda GK, and Clarke K. Gender differences in hypertrophy, insulin resistance and ischemic injury in the aging type 2 diabetic rat heart. *J Mol Cell Cardiol* 2004 **37**: 547-555.

Dudley HW, and Marrian GF. The effect of insulin on the glycogen in the tissues of normal animals. *Biochem J* 1923 **17**: 435-438.

Eguchi M, Kim YH, Kang KW, Shim CY, Jang Y, Dorval T, Kim KJ, and Sweeney G. Ischemia-reperfusion injury leads to distinct temporal cardiac remodeling in normal versus diabetic mice. *PLoS ONE* 2012 **7**: e30450.

Evans G. The glycogen content of the rat heart. *J Physiol* 1934 **82**: 468-480.

Feldmer M, Kaling M, Takahashi S, Mullins JJ, and Ganten D. Glucocorticoid- and estrogen-responsive elements in the 5'-flanking region of the rat angiotensinogen gene. *J Hypertens* 1991 **9**: 1005-1012.

George AM, Jacob AG, and Fogelfeld L. Lean diabetes mellitus: An emerging entity in the era of obesity. *World J Diabetes* 2015 **6**: 613-620.

- Han JC, Goo S, Barrett CJ, Mellor KM, Taberner AJ, and Loiselle DS.** The afterload-dependent peak efficiency of the isolated working rat heart is unaffected by streptozotocin-induced diabetes. *Cardiovasc Diabetol* 2014 **13**: 4.
- He C, Zhu H, Li H, Zou MH, and Xie Z.** Dissociation of bcl-2-beclin1 complex by activated ampk enhances cardiac autophagy and protects against cardiomyocyte apoptosis in diabetes. *Diabetes* 2013 **62**: 1270-1281.
- Huisamen B.** Protein kinase b in the diabetic heart. *Mol Cell Biochem* 2003 **249**: 31-38.
- Hunter RW, Treebak JT, Wojtaszewski JF, and Sakamoto K.** Molecular mechanism by which amp-activated protein kinase activation promotes glycogen accumulation in muscle. *Diabetes* 2011 **60**: 766-774.
- Huynh K, Kiriazis H, Du XJ, Love JE, Jandeleit-Dahm KA, Forbes JM, McMullen JR, and Ritchie RH.** Coenzyme q10 attenuates diastolic dysfunction, cardiomyocyte hypertrophy and cardiac fibrosis in the db/db mouse model of type 2 diabetes. *Diabetologia* 2012 **55**: 1544-1553.
- Jiang S, Heller B, Tagliabracci VS, Zhai L, Irimia JM, DePaoli-Roach AA, Wells CD, Skurat AV, and Roach PJ.** Starch binding domain-containing protein 1/genethonin 1 is a novel participant in glycogen metabolism. *J Biol Chem* 2010 **285**: 34960-34971.
- Jung CH, Ro SH, Cao J, Otto NM, and Kim DH.** Mtor regulation of autophagy. *FEBS Lett* 2010 **584**: 1287-1295.
- Kakinuma Y, Okada S, Nogami M, and Kumon Y.** The human female heart incorporates glucose more efficiently than the male heart. *Int J Cardiol* 2013 **168**: 2518-2521.
- Kanamori H, Takemura G, Goto K, Tsujimoto A, Mikami A, Ogino A, Watanabe T, Morishita K, Okada H, Kawasaki M, Seishima M, and Minatoguchi S.** Autophagic adaptations in diabetic cardiomyopathy differ between type 1 and type 2 diabetes. *Autophagy* 2015 **11**: 1146-1160.
- Kaye DM, and Krum H.** Drug discovery for heart failure: A new era or the end of the pipeline? *Nat Rev Drug Discov* 2007 **6**: 127-139.
- Kim J, Kundu M, Viollet B, and Guan KL.** Ampk and mtor regulate autophagy through direct phosphorylation of ulk1. *Nat Cell Biol* 2011 **13**: 132-141.

Koenig A, Sateriale A, Budd RC, Huber SA, and Buskiewicz IA. The role of sex differences in autophagy in the heart during coxsackievirus b3-induced myocarditis. *J Cardiovasc Transl Res* 2014 **7**: 182-191.

Kokubun E, Hirabara SM, Fiamoncini J, Curi R, and Haebisch H. Changes of glycogen content in liver, skeletal muscle, and heart from fasted rats. *Cell Biochem Funct* 2009 **27**: 488-495.

Kotoulas OB, Kalamidas SA, and Kondomerkos DJ. Glycogen autophagy in glucose homeostasis. *Pathol Res Pract* 2006 **202**: 631-638.

Lajoie C, Calderone A, Trudeau F, Lavoie N, Massicotte G, Gagnon S, and Beliveau L. Exercise training attenuated the pkb and gsk-3 dephosphorylation in the myocardium of zdf rats. *J Appl Physiol* 2004 **96**: 1606-1612.

Lee JH, Lee JH, Jin M, Han SD, Chon GR, Kim IH, Kim S, Kim SY, Choi SB, and Noh YH. Diet control to achieve euglycemia induces significant loss of heart and liver weight via increased autophagy compared with ad libitum diet in diabetic rats. *Exp Mol Med* 2014 **46**: e111.

Lee Y, Hong Y, Lee SR, Chang KT, and Hong Y. Autophagy contributes to retardation of cardiac growth in diabetic rats. *Lab Anim Res* 2012 **28**: 99-107.

Lim HS, MacFadyen RJ, and Lip GY. Diabetes mellitus, the renin-angiotensin-aldosterone system, and the heart. *Arch Intern Med* 2004 **164**: 1737-1748.

Liu JE, Palmieri V, Roman MJ, Bella JN, Fabsitz R, Howard BV, Welty TK, Lee ET, and Devereux RB. The impact of diabetes on left ventricular filling pattern in normotensive and hypertensive adults: The strong heart study. *J Am Coll Cardiol* 2001 **37**: 1943-1949.

Mack HI, Zheng B, Asara JM, and Thomas SM. Ampk-dependent phosphorylation of ulk1 regulates atg9 localization. *Autophagy* 2012 **8**: 1197-1214.

Malfitano C, de Souza Junior AL, Carbonaro M, Bolsoni-Lopes A, Figueroa D, de Souza LE, Silva KA, Consolim-Colombo F, Curi R, and Irigoyen MC. Glucose and fatty acid metabolism in infarcted heart from streptozotocin-induced diabetic rats after 2 weeks of tissue remodeling. *Cardiovasc Diabetol* 2015 **14**: 149.

Mammucari C, Schiaffino S, and Sandri M. Downstream of akt: Foxo3 and mtor in the regulation of autophagy in skeletal muscle. *Autophagy* 2008 **4**: 524-526.

Mansor LS, Gonzalez ER, Cole MA, Tyler DJ, Beeson JH, Clarke K, Carr CA, and Heather LC. Cardiac metabolism in a new rat model of type 2 diabetes using high-fat diet with low dose streptozotocin. *Cardiovasc Diabetol* 2013 **12**: 136.

Marsh SA, Powell PC, Dell'Italia LJ, and Chatham JC. Cardiac o-glucosylation blunts autophagic signaling in the diabetic heart. *Life sciences* 2013 **92**: 648-656.

Maselli A, Matarrese P, Straface E, Canu S, Franconi F, and Malorni W. Cell sex: A new look at cell fate studies. *Faseb j* 2009 **23**: 978-984.

Mellor KM, Bell JR, Young MJ, Ritchie RH, and Delbridge LM. Myocardial autophagy activation and suppressed survival signaling is associated with insulin resistance in fructose-fed mice. *J Mol Cell Cardiol* 2011 **50**: 1035-1043.

Mellor KM, Curl CL, Chandramouli C, Pedrazzini T, Wendt IR, and Delbridge LM. Ageing-related cardiomyocyte functional decline is sex and angiotensin ii dependent. *Age (Dordr)* 2014 **36**: 9630.

Moore A, Shindikar A, Fomison-Nurse I, Riu F, Munasinghe PE, Ram TP, Saxena P, Coffey S, Bunton RW, Galvin IF, Williams MJ, Emanuelli C, Madeddu P, and Kataré R. Rapid onset of cardiomyopathy in stz-induced female diabetic mice involves the downregulation of pro-survival pim-1. *Cardiovasc Diabetol* 2014 **13**: 68.

Mowry RW, and Bangle R, Jr. Histochemically demonstrable glycogen in the human heart, with special reference to glycogen storage disease and diabetes mellitus. *Am J Pathol* 1951 **27**: 611-625.

Munasinghe PE, Riu F, Dixit P, Edamatsu M, Saxena P, Hamer NS, Galvin IF, Bunton RW, Lequeux S, Jones G, Lamberts RR, Emanuelli C, Madeddu P, and Kataré R. Type-2 diabetes increases autophagy in the human heart through promotion of beclin-1 mediated pathway. *Int J Cardiol* 2016 **202**: 13-20.

Myers RB, Fomovsky GM, Lee S, Tan M, Wang BF, Patwari P, and Yoshioka J. Deletion of thioredoxin-interacting protein improves cardiac inotropic reserve in the streptozotocin-induced diabetic heart. *Am J Physiol Heart Circ Physiol* 2016 **310**: H1748-1759.

Nishida K, Kyo S, Yamaguchi O, Sadoshima J, and Otsu K. The role of autophagy in the heart. *Cell Death Differ* 2009 **16**: 31-38.

Palomeque J, Delbridge L, and Petroff MV. Angiotensin ii: A regulator of cardiomyocyte function and survival. *Front Biosci (Landmark Ed)* 2009 **14**: 5118-5133.

Patten RD, Pourati I, Aronovitz MJ, Baur J, Celestin F, Chen X, Michael A, Haq S, Nuedling S, Grohe C, Force T, Mendelsohn ME, and Karas RH. 17beta-estradiol reduces cardiomyocyte apoptosis in vivo and in vitro via activation of phospho-inositide-3 kinase/akt signaling. *Circ Res* 2004 **95**: 692-699.

Pei XM, Yung BY, Yip SP, Chan LW, Wong CS, Ying M, and Siu PM. Protective effects of desacyl ghrelin on diabetic cardiomyopathy. *Acta Diabetol* 2015 **52**: 293-306.

Pellerin I, Vuillermoz C, Jouvenot M, Ordener C, Royez M, and Adessi GL. Identification and characterization of an early estrogen-regulated rna in cultured guinea-pig endometrial cells. *Mol Cell Endocrinol* 1993 **90**: R17-21.

Peterson LR, Herrero P, Coggan AR, Kisrieva-Ware Z, Saeed I, Dence C, Koudelis D, McGill JB, Lyons MR, Novak E, Davila-Roman VG, Waggoner AD, and Gropler RJ. Type 2 diabetes, obesity, and sex difference affect the fate of glucose in the human heart. *Am J Physiol Heart Circ Physiol* 2015 **308**: H1510-1516.

Pham T, Loisel D, Power A, and Hickey AJ. Mitochondrial inefficiencies and anoxic atp hydrolysis capacities in diabetic rat heart. *Am J Physiol Cell Physiol* 2014 **307**: C499-507.

Piekarski A, Khaldi S, Greene E, Lassiter K, Mason JG, Anthony N, Bottje W, and Dridi S. Tissue distribution, gender- and genotype-dependent expression of autophagy-related genes in avian species. *PLoS One* 2014 **9**: e112449.

Porrello ER, D'Amore A, Curl CL, Allen AM, Harrap SB, Thomas WG, and Delbridge LM. Angiotensin ii type 2 receptor antagonizes angiotensin ii type 1 receptor-mediated cardiomyocyte autophagy. *Hypertension* 2009a **53**: 1032-1040.

Porrello ER, and Delbridge LM. Cardiomyocyte autophagy is regulated by angiotensin ii type 1 and type 2 receptors. *Autophagy* 2009b **5**: 1215-1216.

Raben N, Baum R, Schreiner C, Takikita S, Mizushima N, Ralston E, and Plotz P. When more is less: Excess and deficiency of autophagy coexist in skeletal muscle in pompe disease. *Autophagy* 2009 **5**: 111-113.

Rabinowitz M. Control of metabolism and synthesis of macromolecules in normal and ischemic heart. *J Mol Cell Cardiol* 1971 **2**: 277-292.

Reichelt ME, Mellor KM, Curl CL, Stapleton D, and Delbridge LM. Myocardial glycophagy - a specific glycogen handling response to metabolic stress is accentuated in the female heart. *J Mol Cell Cardiol* 2013 **65**: 67-75.

Russo SB, Baicu CF, Van Laer A, Geng T, Kasiganesan H, Zile MR, and Cowart LA. Ceramide synthase 5 mediates lipid-induced autophagy and hypertrophy in cardiomyocytes. *J Clin Invest* 2012 **122**: 3919-3930.

Sakakibara M, Hirashiki A, Cheng XW, Bando Y, Ohshima K, Okumura T, Funahashi H, Ohshima S, and Murohara T. Association of diabetes mellitus with myocardial collagen accumulation and relaxation impairment in patients with dilated cardiomyopathy. *Diabetes Res Clin Pract* 2011 **92**: 348-355.

Shearer J, Ross KD, Hughey CC, Johnsen VL, Hittel DS, and Severson DL. Exercise training does not correct abnormal cardiac glycogen accumulation in the db/db mouse model of type 2 diabetes. *Am J Physiol Endocrinol Metab* 2011 **301**: E31-39.

Straface E, Vona R, Gambardella L, Ascione B, Marino M, Bulzomi P, Canu S, Coinu R, Rosano G, Malorni W, and Franconi F. Cell sex determines anoikis resistance in vascular smooth muscle cells. *FEBS Lett* 2009 **583**: 3448-3454.

van Heerebeek L, Hamdani N, Handoko ML, Falcao-Pires I, Musters RJ, Kupreishvili K, Ijsselmuiden AJ, Schalkwijk CG, Bronzwaer JG, Diamant M, Borbely A, van der Velden J, Stienen GJ, Laarman GJ, Niessen HW, and Paulus WJ. Diastolic stiffness of the failing diabetic heart: Importance of fibrosis, advanced glycation end products, and myocyte resting tension. *Circulation* 2008 **117**: 43-51.

Velloso LA, Folli F, Perego L, and Saad MJ. The multi-faceted cross-talk between the insulin and angiotensin ii signaling systems. *Diabetes Metab Res Rev* 2006 **22**: 98-107.

Wang B, Yang Q, Sun YY, Xing YF, Wang YB, Lu XT, Bai WW, Liu XQ, and Zhao YX. Resveratrol-enhanced autophagic flux ameliorates myocardial oxidative stress injury in diabetic mice. *J Cell Mol Med* 2014 **18**: 1599-1611.

Warren S. The effect of insulin on pathologic glycogen deposits in diabetes mellitus. *Am J Med Sci* 1930 **179** pp. 482-488.

Xie Z, He C, and Zou MH. Amp-activated protein kinase modulates cardiac autophagy in diabetic cardiomyopathy. *Autophagy* 2011a **7**: 1254-1255.

Xie Z, Lau K, Eby B, Lozano P, He C, Pennington B, Li H, Rathi S, Dong Y, Tian R, Kem D, and Zou MH. Improvement of cardiac functions by chronic metformin treatment is associated with enhanced cardiac autophagy in diabetic ove26 mice. *Diabetes* 2011b **60**: 1770-1778.

Xu X, Hua Y, Nair S, Zhang Y, and Ren J. Akt2 knockout preserves cardiac function in high-fat diet-induced obesity by rescuing cardiac autophagosome maturation. *J Mol Cell Biol* 2013a **5**: 61-63.

Xu X, Kobayashi S, Chen K, Timm D, Volden P, Huang Y, Gulick J, Yue Z, Robbins J, Epstein PN, and Liang Q. Diminished autophagy limits cardiac injury in mouse models of type 1 diabetes. *J Biol Chem* 2013b **288**: 18077-18092.

Xu X, and Ren J. Macrophage migration inhibitory factor (mif) knockout preserves cardiac homeostasis through alleviating akt-mediated myocardial autophagy suppression in high-fat diet-induced obesity. *Int J Obes (Lond)* 2015 **39**: 387-396.

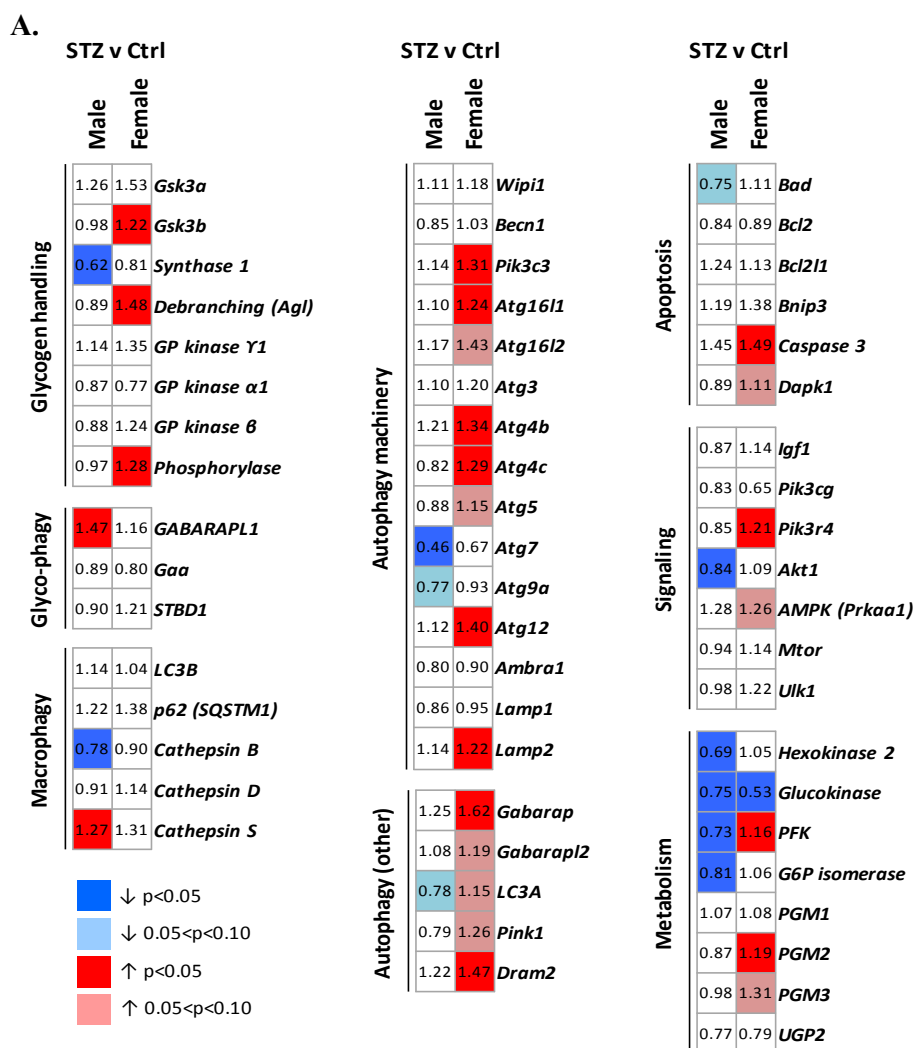
Zhao Y, Zhang L, Qiao Y, Zhou X, Wu G, Wang L, Peng Y, Dong X, Huang H, Si L, Zhang X, Zhang L, Li J, Wang W, Zhou L, and Gao X. Heme oxygenase-1 prevents cardiac dysfunction in streptozotocin-diabetic mice by reducing inflammation, oxidative stress, apoptosis and enhancing autophagy. *PLoS One* 2013 **8**: e75927.

Zou MH, and Xie Z. Regulation of interplay between autophagy and apoptosis in the diabetic heart: New role of ampk. *Autophagy* 2013 **9**: 624-625.

Table 5.1 Systemic characteristics of diabetic rodent models vs. controls.

	Lean T1D (STZ male)	Lean T1D (STZ female)	Lean T2D (Fructose-fed)	Obese T2D (db/db)
Blood glucose	↑↑	↑	↑	↑↑
Body weight	↓	↔	↓	↑↑
GTT AUC	↑	↑	↑	?
Plasma insulin	↓↓	↓	↔	↑
Cardiac weight index (mg/g)	↔	↔	↔	↓

Arrow directions are indicative of data reported in Chapter 3 (STZ male and female mice), and previously published data (Bowden *et al.*, 2015; Mellor *et al.*, 2011).



B.

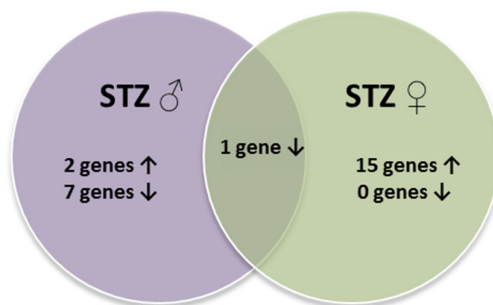
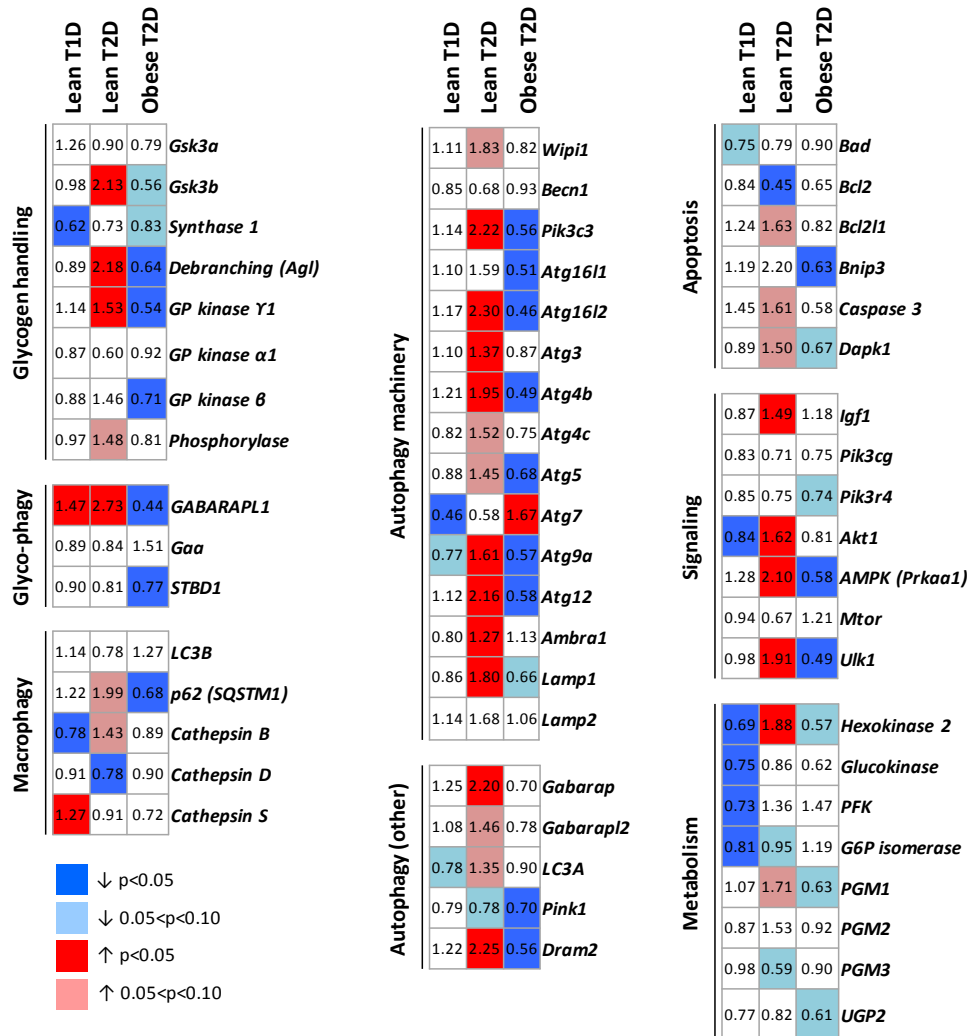


Figure 5.1. Sex differences in energy utilisation and cell death pathways in T1D mice. **A.** Stylised heatmap summarising trends in mRNA expression (relative to reference gene) in male and female STZ mouse hearts, red: increase, p<0.05; pink: increase, 0.05<p<0.1; blue: decrease, p<0.05; pale blue: decrease, 0.05<p<0.1, relative to respective control for male and female. **B.** Venn diagram of significantly up or down regulated genes relative to control for male vs. female STZ mouse hearts.

A.



B.

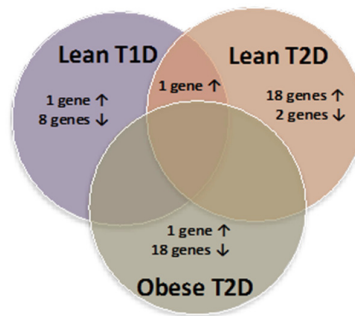
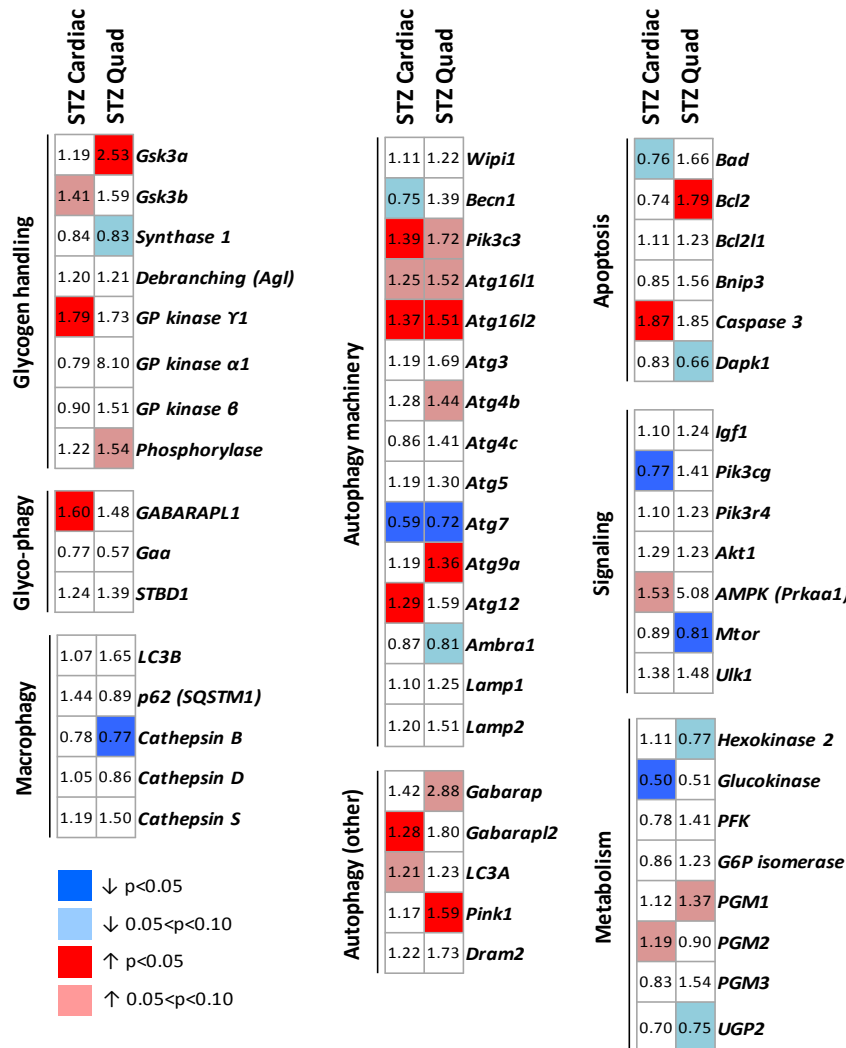


Figure 5.2. Energy utilisation and cell death pathway gene expression responses in T1D and T2D male mice. **A.** Stylised heatmap summarising trends in mRNA expression (relative to reference gene) in male T1D (STZ), insulin resistant lean (fructose-fed) and T2D obese (*db/db*) mouse hearts. Red: increase, $p < 0.05$; pink: increase, $0.05 < p < 0.1$; blue: decrease, $p < 0.05$; pale blue: decrease, $0.05 < p < 0.1$, relative to respective control. **B.** Venn diagram of significantly up or down regulated genes relative to control for male T1D (STZ), insulin resistant T2D lean (fructose-fed) and T2D obese (*db/db*) mouse hearts.

A.



B.

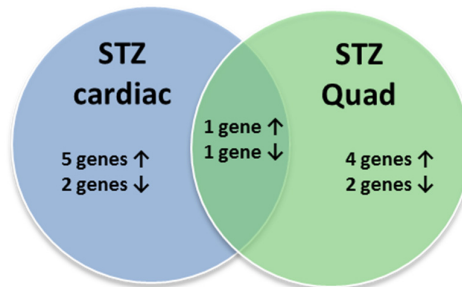


Figure 5.3. Energy utilisation and cell death pathway in cardiac vs. skeletal muscle of T1D male mice. **A.** Stylised heatmap summarising trends in mRNA expression (relative to reference gene) in acute male T1D mouse hearts and quadriceps muscle. Red: increase, p < 0.05; pink: increase, 0.05 < p < 0.1; blue: decrease, p < 0.05; pale blue: decrease, 0.05 < p < 0.1, relative to respective control. **B.** Venn diagram of significantly up or down regulated genes relative to control for male acute insulin withdrawn (1week STZ) mouse hearts and quadriceps muscle.

CHAPTER 6

Type-1 diabetic cardiac functional and structural pathology is exacerbated by elevated cardiac AngII in male but not female mice

Manuscript generated from combined datasets of Chapter 4 and Chapter 6:

Chandramouli C*, Reichelt ME*, Bienvenu LA, Curl CL, Ritchie RH, Mellor KM[#], Delbridge LMD. [#] Type-1-diabetic cardiac functional and structural pathology is exacerbated by elevated cardiac angiotensin II in male but not female mice. *Cardiovasc Diabetol*. [In preparation]

6.1 Introduction

Diabetic cardiomyopathy is characterised by left ventricular dysfunction, and has been associated with increased activation of the renin-angiotensin system (RAS) in diabetic patients (Frustaci *et al.*, 2000) and rodent models (Farah *et al.*, 2007; Kamide *et al.*, 2002; Singh *et al.*, 2008). Inhibition of RAS is a key therapeutic measure in diabetic patients (Rodriguez *et al.*, 2012) and this results in improved systolic and diastolic function in diabetic rodents (Masuda *et al.*, 2012). Possible cross-talk between insulin and Angiotensin II (AngII) signalling has been suggested, as AngII has been implicated in adversely affecting glucose metabolism and reducing insulin-mediated glucose uptake in diabetes (Cooper *et al.*, 2007; Luther *et al.*, 2011). Sex differences in responses to RAS perturbation in non-cardiac tissues (Sullivan, 2008) and a trend towards lower efficacy of RAS inhibitors in females (Rabi *et al.*, 2008) have been reported. The findings presented in Chapters 3 and 4 in this thesis demonstrate a greater functional decline with T1D, associated with a selective glycogen handling response and an increased autophagic drive in female compared to male T1D hearts. This chapter extends this work to investigate sex differences in diabetic functional pathology and ischemic resilience in an elevated cardiac AngII setting.

Upregulation of cardiac AngII has been implicated as a key contributor to underlying diastolic dysfunction and fibrotic remodelling in diabetes (Benazzoug *et al.*, 1998; Connelly *et al.*, 2007; Singh *et al.*, 2008). Clinical usage of RAS inhibitors (ACEi and ARBs) has shown beneficial effects through reduced oxidative damage and improved endothelial function. Studies utilising RAS inhibitors in diabetes report improved diastolic function (increased E/A ratio) in STZ-induced T1D male mice (Yong *et al.*, 2013). Yet, RAS inhibitors have limited efficacy on intracellular AngII effects in the heart (Baker *et al.*, 2006). Immunohistochemical analysis of cardiac tissue from diabetic non-hypertensive patients exhibited 3.4-fold higher AngII levels (*vs.* non-diabetic patients). Interestingly, diabetic hypertensive patients exhibited 2-fold higher cardiac AngII levels (*vs.* non-hypertensive diabetic patients), despite being treated with ACE inhibitors (Frustaci *et al.*, 2000). Whether the lowering of fold increase is due to the actions of ACE inhibitors primarily on non-cardiac AngII to reduce hypertension (pre-load) or on intracardiac AngII is yet to be confirmed. A role for systemic RAS in diabetic cardiopathology is evident but the specific role of cardiac AngII is not fully understood.

All components of the RAS are expressed in the heart and a role for local AngII in functional and structural pathology has been demonstrated (Dostal *et al.*, 1992). Mice with cardiac-specific angiotensinogen (*AOGN*) gene overexpression exhibit increased intracardiac AngII levels and unchanged plasma AngII levels (Mazzolai *et al.*, 2000). Progression towards failure involves a transition from cardiac hypertrophy (~20 weeks age) to a decompensated, dilated phenotype associated with severe contractile abnormalities (~50 weeks age), in the absence of increased workload/hypertension (Domenighetti *et al.*, 2005; Gusev *et al.*, 2009). *In vitro* studies from these AngII-Tg mice have shown that autophagy in neonatal cardiomyocytes is modulated by local cardiac AngII (Porrello *et al.*, 2009). Hence, investigating the direct effects of local intracardiac AngII on the diabetic heart in isolation from systemic effects of AngII is possible with this model.

Some evidence of sex differences in AngII modulation have been reported both clinically and experimentally. A consistent trend towards greater benefit of ACE inhibitors in men than women was reported in a meta-analysis of clinical trials evaluating the efficacy of ACE inhibitors and ARBs (Rabi *et al.*, 2008). Experimentally, sex differences have also been reported in the expression of various components of RAS (“classical” and “non-classical”) in non-diabetic cardiac pathology (Dalpiaz *et al.*, 2015). Estrogen has also been shown to modulate various components of RAS. An estrogen response element located at the promoter region of the *AOGN* gene may be instrumental in regulating local tissue AngII production (Feldmer *et al.*, 1991). Female vulnerability in cardiomyocyte contractile deficit with age under an elevated cardiac AngII setting has been reported (Mellor *et al.*, 2014) in a non-diabetic setting. No study to date has investigated sex differences in functional changes in the T1D heart under an elevated intracardiac AngII setting, independent of hemodynamic loading.

Given that (i) cardiac RAS is upregulated in clinical and experimental diabetic settings, (ii) clinical efficacy of RAS inhibitors is lower in women, and (iii) estrogen has a regulatory role on RAS components, it was hypothesised that cardiac AngII excess would further exacerbate cardiac functional decline in T1D females. To test this hypothesis, STZ treated mice with cardiac-specific *AOGN* overexpression were assessed for *in vivo* echocardiographic function and were subjected to an *ex vivo* ischemia-reperfusion protocol. A worsened T1D cardiac functional phenotype and “loss

of benefit” in reperfusion recovery following ischemia was evident in male AngII-Tg mice. Surprisingly, diabetic female hearts exhibited a similar functional pathology with and without AngII upregulation.

6.2 Specific Methodology

6.2A Animal model and STZ induction

The transgenic mouse line with cardiac overexpression of the AOGN gene (Tg1306/IR) was used in experiments presented in this chapter. Details of transgenic line development have been described in Chapter 2 Section 2.3. Both male and female AngII-Tg mice (n=9-10/group) were induced with T1D at 15 weeks of age via 5 daily 55 mg/kg STZ injections as described in Chapter 2 Section 2.4. In agreement with previous reports on hypertrophy in this AngII-Tg mice model (Domenighetti *et al.*, 2005; Mazzolai *et al.*, 1998), AngII-induced cardiac hypertrophy was evident as observed from increased cardiac weight indices in both sexes (Male-Tg *vs.* Wildtype: 25% increase, Female-Tg *vs.* Wildtype: 14% increase, $p < 0.05$, Table 3.2 and Table 6.1).

6.2B *In vivo* echocardiographic functional assessment

Following 7 weeks of diabetes induction, cardiac functional and ventricular wall dimensional parameters were measured using transthoracic echocardiography (Vivid E9, GE Healthcare, CT, USA) in mice under light anaesthesia with tribromethanol (Dosage: 2.5%, 0.01ml/g i.p) as described in Chapter 2 Section 2.13.

6.2C *Ex vivo* isolated heart functional assessment

Hearts were retrogradely perfused on a Langendorff rig at a constant pressure of 80mmHg for 30 mins prior to 20 mins of global no-flow ischemia and 45 mins of reperfusion as described in Chapter 2 Section 2.18. Baseline function was measured prior to 20 mins ischemia and recovery was assessed at 30 mins during reperfusion.

6.3 Results

To determine whether elevated cardiac AngII modulated the sex-specific cardiac response to T1D, *in vivo* functional changes and *ex vivo* ischemic vulnerability was investigated between Ctrl and STZ groups in AngII-Tg mice of both sexes. Data from Wildtype T1D male and female, presented in Chapters 3 and 4, have been included for comparative purposes.

6.3A *In vivo* diastolic dysfunction is evident in male and female T1D mice with elevated cardiac AngII

The T1D systemic effects observed in Chapters 3 and 4 in Wildtype mice were similar in AngII-Tg mice (Table 6.1). The STZ-induced increase in blood glucose was less pronounced in females than males (Male-Tg STZ vs. Ctrl: 2.7 fold and Female-Tg STZ vs. Ctrl: 1.7 fold increase, $p < 0.05$, Table 6.1), confirming that cardiac-specific AngII overexpression had no systemic effects. With STZ-induction, cardiac weight indices (heart weight normalised to tibia length) were significantly reduced in males (Male-Tg Ctrl: 10.5 ± 0.5 vs. STZ: 8.3 ± 0.3 mg/mm) while remaining unchanged in females (Female-Tg Ctrl: 7.8 ± 0.2 vs. STZ: 7.0 ± 0.2 mg/mm, Table 6.1) in AngII-Tg mice.

In the Wildtype mice, STZ-induced diastolic dysfunction was evident in females and unchanged in males (Chapter 3, Figure 3.3). To determine if upregulation of cardiac AngII exacerbates the diastolic dysfunction observed in diabetes (Chapter 3 Figure 3.3C), apical 4-chamber echocardiography was performed in Ctrl and STZ groups of AngII-Tg mice in both sexes. Representative traces of pulse wave Doppler (Figure 6.1A) and tissue Doppler (Figure 6.1B) suggest an impaired relaxation filling pattern in both sexes with STZ administration. T1D-induced diastolic dysfunction in AngII-Tg mice was observed in both sexes as evidenced by the increased E/E' ratio (~35% increase STZ vs. Ctrl, ANOVA STZ factor effect, $p < 0.05$, Figure 6.1C). Mitral valve deceleration time (Dec T) showed a tendency towards an increase in males (~40% increase in Male-Tg STZ vs. Ctrl, $p = 0.07$, Figure 6.1D) and was significantly increased in females (77% increase in Female-Tg STZ vs. Ctrl, $p < 0.05$, Figure 6.1D). Therefore, T1D-induced impaired relaxation was evident in both sexes in AngII-Tg mice and diastolic dysfunction emerged with elevated AngII in males.

6.3B *In vivo* systolic dysfunction is not evident in male and female T1D mice with elevated cardiac AngII

In Wildtype mice, systolic function was preserved (ejection fraction >60%, Chapter 3, Figure 3.4) with STZ in both sexes, despite a modest decline in females (Chapter 3 Figure 3.4). To assess whether sex differences in contractile functional response to T1D are more marked in mice with cardiac AngII overexpression, ejection fraction and fractional shortening were measured in STZ male and female AngII-Tg mice. STZ treatment in AngII-Tg mice did not cause any marked changes in ejection fraction in males (Male-Tg Ctrl: $68.1 \pm 3.7\%$ vs. STZ: $60.8 \pm 3.3\%$, $p = \text{ns}$, Figure 6.2A) and females (Female-Tg Ctrl: $67.2 \pm 2.4\%$ vs. STZ: $65.0 \pm 1.8\%$, $p = \text{ns}$, Figure 6.2A). Similarly, STZ-induced tendency for reduction of fractional shortening in AngII-Tg mice of both sexes was observed, although not significant (Male-Tg STZ vs. Ctrl: 15% and Female-Tg STZ vs. Ctrl: 6% increase, $p = \text{ns}$, Figure 6.2B). Thus, systolic function was relatively well preserved following chronic STZ treatment in both sexes in AngII-Tg mice, similar to that observed in Wildtype mice.

6.3C Left ventricular dilation and wall thinning from echocardiography is evident in male but not female T1D mice with elevated cardiac AngII

As presented in Chapter 3, T1D induced left ventricular septal and posterior wall thinning in both sexes in Wildtype mice. To assess whether sex differences in the morphological response to T1D are more accentuated in mice with cardiac AngII overexpression, left ventricular wall dimensions were measured in AngII-Tg mice of both sexes from *m-mode* images. In AngII-Tg males, STZ induced an increase in chamber diameter at diastole (Male-Tg STZ vs. Ctrl: 10.8% increase, $p < 0.05$, Figure 6.3B) and a reduction in posterior wall (Male-Tg STZ vs. Ctrl: 31.3% decrease, $p < 0.05$, Figure 6.3C) as well as a reduction in septal wall thickness (Male-Tg STZ vs. Ctrl: 17.9% decrease, $p < 0.05$, Table 6.2). In contrast to the STZ-induced wall thinning observed in Female-Wildtype (Figure 3.5C), wall parameters were unchanged with STZ in Female-Tg mice (no differences in chamber diameter and posterior wall thickness, Figure 6.3B-C). Some sex differences were evident in the non-diabetic AngII-Tg mice, as shown by lower posterior wall thickness in females (Tg-Ctrl females vs. males: 16.9% lower, $p < 0.05$, Table 6.3C) and some sex differences became apparent with STZ, as evidenced by smaller chamber diameter in STZ females (Tg-STZ females vs. males:

16.7% lower, $p < 0.05$, Figure 6.3B). Thus, left ventricular dilation and wall thinning was observed with STZ in male but not female mice with increased cardiac AngII.

6.3D *Ex vivo* female ischemic injury in T1D mice with elevated cardiac AngII is less severe than in male mice

Similar to observations in Wildtype (Chapter 4, Figure 4.1), T1D did not have an effect on *ex vivo* baseline function measured during the equilibration period prior to ischemia in either sex under the elevated cardiac AngII setting (Table 6.3). In Wildtype mice, T1D-induced earlier onset of contracture in females while peak contracture remained unchanged (Chapter 4, Figure 4.2C-D). No T1D-induced differences were observed in Wildtype males in either parameter (Chapter 4, Figure 4.2C-B). To establish whether sex differences in ischemic injury response to T1D are accentuated in mice with cardiac AngII overexpression, time of onset of contracture and peak contracture were measured in STZ AngII-Tg isolated mouse hearts of both sexes during no-flow ischemia. In males, time to contracture was significantly reduced with STZ in AngII-Tg (Male-Tg Ctrl: 14.2 ± 0.8 vs. STZ: 10.8 ± 0.8 mins, $p < 0.05$, Figure 6.4C) but was not evident in Wildtype (Figure 4.2C). In females, in contrast to the earlier onset of contracture with STZ in Wildtype (Figure 4.2C), the onset of contracture was not different with STZ in female AngII-Tg mice (Female-Tg Ctrl: 16.7 ± 0.5 vs. STZ: 15.1 ± 1.9 mins, Figure 6.4C). This suggests differential cardiac AngII effects in both sexes. Similarly, peak contracture was not different with STZ in males or females with increased cardiac AngII (Figure 6.4D). Sex differences were evident with earlier onset of contracture with STZ in males (Tg-STZ male vs. females 40.1% quicker, $p < 0.05$ Figure 6.4C) and increased extent of ischemic injury, as observed from increased peak contracture (Tg-STZ male vs. females 1.9-fold higher, $p < 0.05$ Figure 6.4D). Other reperfusion injury may involve ventricular fibrillation (VF), ventricular tachycardia (VT) and LDH release with loss in sarcolemmal integrity but were not measured in this study. T1D-induced greater ischemic injury in males while the effects were less pronounced in female T1D in cardiac AngII-Tg mice.

6.3E No effect of T1D on contractile recovery in isolated hearts post-ischemia in male and female mice with elevated cardiac AngII

In Wildtype mice, STZ-induced greater *ex vivo* post-ischemic recovery in males and no change in females (Chapter 4, Figure 4.4). To evaluate whether sex differences in

ischemic response to T1D are accentuated in mice with cardiac AngII overexpression, functional recovery during reperfusion (expressed as %, normalised to pre-ischemia) was measured in isolated hearts of STZ male and female AngII-Tg mice. Despite greater ischemic injury in male STZ, there was no difference in the extent of post-ischemic functional recovery (expressed as % baseline) with elevated cardiac AngII. In contrast to STZ-induced greater functional recovery in male wildtype (Chapter 4, Figure 4.4), developed pressure (Male-Tg Ctrl: 69.3 ± 8.5 vs. STZ: $73.3 \pm 5.5\%$, Figure 6.4C), $dP/dt_{\max}$ (Male-Tg Ctrl: 85.4 ± 8.1 vs. STZ: $90.4 \pm 5.4\%$, Figure 6.4D) and $dP/dt_{\min}$ (Male-Tg Ctrl: 80.4 ± 7.2 vs. STZ: $77.9 \pm 9.3\%$, Figure 6.4C) remained unchanged with STZ at 30 mins reperfusion in males in AngII-Tg mice. Similar to the STZ effect in female wildtype (Chapter 4 Figure 4.4), STZ did not have any effect on cardiac functional recovery during reperfusion after ischemia in AngII-Tg mice. No STZ-induced differences were observed in developed pressure (Female-Tg Ctrl: 58.8 ± 5.7 vs. STZ: $55.7 \pm 9.3\%$, Figure 6.4C), $dP/dt_{\max}$ (Female-Tg Ctrl: 71.8 ± 6.7 vs. STZ: $62.9 \pm 11.0\%$, Figure 6.4D) and $dP/dt_{\min}$ (Female-Tg Ctrl: 62.1 ± 7.9 vs. STZ: $58.0 \pm 12.7\%$, Figure 6.4C). With the exception of a 30.4% higher $dP/dt_{\max}$ recovery in Tg-STZ males (vs. females, Figure 6.4D), no other sex differences were detected. STZ did not affect post-ischemic function of both sexes under the elevated cardiac AngII setting.

6.4 Discussion

This is the first study to show diabetes-induced cardiac dysfunction is exacerbated in a setting of high cardiac AngII in males but not females. In males, T1D-induced diastolic dysfunction was only evident in mice with AngII overexpression, but in females, diastolic dysfunction evident in T1D wildtype mice was not altered by AngII upregulation. In AngII-Tg males, ischemic injury was more pronounced with T1D and “*recovery benefit*” observed in Wildtype mice was absent during reperfusion when cardiac AngII was elevated. In females, STZ-induced ischemic injury or contractile changes post-ischemia were not apparent. With elevated AngII, T1D-induced diastolic dysfunction was present in both sexes while wall-thinning pathology was evident only in male T1D. Estrogen regulation of AngII levels locally in the heart could perhaps explain the differential effects in female T1D. Further investigation of crosstalk between estrogen, AngII and insulin signalling in T1D hearts is warranted.

6.4A Exacerbated male T1D-induced *in vivo* diastolic dysfunction with high cardiac AngII

Diastolic dysfunction, preceding the onset of systolic functional deficit, is characteristic of the diabetic heart (Boyer *et al.*, 2004; Zabalgaitia *et al.*, 2001). The cardiac-specific AngII-Tg mice have been demonstrated to develop cardiac hypertrophy at 15-20 weeks, which progresses towards decompensated cardiac failure with diastolic dysfunction ($\sim 45\%$ reduction $dP/dt_{\max}$ and $dP/dt_{\min}$) at 50-60 weeks (Domenighetti *et al.*, 2005). In the present study, STZ-induced diastolic dysfunction was observed in both sexes under high cardiac AngII setting ($\sim 35\%$ increase in E/E' ratio) (Figure 6.1A). Interestingly, the extent of the STZ-induced increase in E/E' ratio and Dec T were more pronounced in male-Tg compared to Wildtype (Figure 3.3C), suggesting an exacerbation of relaxation impairment with high cardiac AngII in males. This is consistent with a study reporting marked STZ-induced diastolic dysfunction in AT_{1a}-KO mice, which displayed elevated intracardiac AngII levels (Yong *et al.*, 2013). Intracellular renin inhibition has also managed to reverse diastolic dysfunction in STZ mice by blunting the increase in cardiac AngII levels (Thomas *et al.*, 2013). In contrast to the preserved systolic function reported in this study (Figure 6.2), STZ-induced reduction in ejection fraction is shown in AT_{1a}-KO mice (Yong *et al.*, 2013), possibly owing to the longer duration of STZ treatment undertaken in that study. Thus, STZ-induced diastolic dysfunction was

evident in both sexes in high cardiac AngII and was exacerbated in males, when compared to Wildtype.

The STZ-induced diastolic functional deficit in AngII-Tg mice was also associated with cardiac structural changes. Ventricular dilation and reduced posterior wall thickness was evident with STZ treatment in male AngII-Tg mice (vs. male AngII-controls) (Figure 6.3). In addition, STZ-induced reduction (~20%) in cardiac weight indices was also apparent in high cardiac AngII (Table 6.2). Again, this contrasts with STZ mice exhibiting no changes in cardiac dimensions, as observed from no differences in chamber diameter or wall thickness in AT_{1a}-KO mice (Yong *et al.*, 2013). This is probably due to the characteristic of AT_{1a}-KO mice, in which cardiac structure changes are not observed (Harada *et al.*, 1998; Sugaya *et al.*, 1995). Perhaps, this effect was more apparent through direct manipulation of the intracellular AngII level in the AngII-Tg mice model utilised in this study (Mazzolai *et al.*, 2000). The STZ-induced exacerbation of diastolic dysfunction was associated with male-specific structural deficit in an elevated cardiac AngII setting.

6.4B Loss of T1D-induced “ischemic benefit” in diabetic male isolated hearts with high AngII

Cardiomyocyte calcium (Ca²⁺) loading is recognised as a major contributor in acute ischemia–reperfusion injury and sex differences have been documented in ischemic resilience (Bell *et al.*, 2011). The cardiac AngII-Tg mice utilised in this study display contractile abnormalities and altered intracellular Ca²⁺ dynamics as early as 15 weeks of age (Domenighetti *et al.*, 2005; Mellor *et al.*, 2014). Therefore, a greater STZ-induced ischemic insult and poorer reperfusion recovery was anticipated with elevated cardiac AngII. As expected, the STZ-induced ischemic insult was greater than in Wildtype, as evidenced from the earlier onset of contracture (~24% quicker) (Figure 6.4C) while peak contracture was unchanged (Figure 6.4D) in males. In contrast to the greater reperfusion recovery observed in Wildtype, in AngII-Tg male mice, no differences were observed in STZ-induced post-ischemic contractile recovery. Developed pressure, dP/dt_{max} and dP/dt_{min} (Figure 6.5) remained unchanged with STZ despite the increased STZ-induced injury during ischemia in the high AngII setting (Figure 6.5). This AngII-induced “loss of STZ ischemic benefit” in *ex vivo* reperfusion is consistent with the

exacerbated STZ-induced *in vivo* diastolic dysfunction observed in male AngII-Tg mice.

In female AngII-Tg mice, no differences in STZ-induced ischemic injury or altered reperfusion recovery were observed. These findings were similar to STZ effects in Wildtype females with the exception of one parameter, STZ-induced earlier onset of contracture in Wildtype. This was surprising given the anticipated effect of estrogen-mediated exacerbation of T1D recovery following ischemia in the high AngII environment. The reason for the lack of differences is unclear and will require further mechanistic evidence. Perhaps, further investigations in the regulatory role of estrogen on different components of RAS (Feldmer *et al.*, 1991) under diabetic settings could provide more insights into these differences. Some physiological and pathophysiological evidence of estrogen modulation of RAS is available from the literature. Non-diabetic cardiomyocytes overexpressing cardiac AngII showed reduced cardiomyocyte percentage shortening and delayed clearance of Ca^{2+} from cytosol in aged female mice (where gonadal production of estrogen is low) (Mellor *et al.*, 2014). One study from the literature examined the effects of elevated cardiac RAS in ovariectomised female T2D (10% high-fructose, *w/v* in solution for 9 weeks) hearts. They reported that estradiol supplementation blunted the T2D-induced increase in mRNA and protein expression of ACE and AT₁R in ovariectomised mice hearts (Bundalo *et al.*, 2015). Unfortunately, functional differences were not reported in this study, making it difficult to interpret whether the shift in gene expression was adaptive or maladaptive (Bundalo *et al.*, 2015). Thus, female T1D hearts overexpressing cardiac AngII displayed no changes in post-ischemic function, similar to observations in Wildtype. It would be informative to measure AngII receptor levels in all groups to identify whether STZ exacerbates intracardiac AngII levels in AngII-Tg mice.

As expected, cardiac-specific overexpression of AngII did not further exacerbate the systemic response to T1D. Comparable to T1D effects in Wildtype, the STZ-induced increase in blood glucose was less pronounced in females compared to male mice (Table 6.1). Previous reports on blood pressure measurements, via intra-arterial catheter, in the current model of AngII-Tg mice suggested a normotensive status (Mazzolai *et al.*, 1998). Therefore, the diabetic systemic insult was not affected by

cardiac AngII overexpression and the changes observed in the heart were independent of hemodynamic loading.

In summary, STZ-induced deterioration in function in both sexes and structural deficit in males but not females was observed when cardiac AngII was elevated. It is possible that a local estrogen-AngII interaction may be operational in female diabetic hearts, providing a fundamental mechanistic basis for differential effects of RAS inhibitors in both sexes. More detailed evaluation of AngII levels, sex steroid levels, glycogen content and cell death induction in both sexes would be informative in explaining this differential diabetic susceptibility in the elevated cardiac AngII setting.

6.5 Conclusions

A female disadvantage in functional pathology in diabetic cardiomyopathy, despite less pronounced systemic changes, was established in Chapter 3 of this thesis. The two subsequent chapters (Chapter 4 and 5) delineated sex-specific glycogen handling in stress and differences in progression towards cell death. Given the link between estrogen and RAS components, this chapter attempted to investigate the role of local cardiac AngII in exacerbating T1D functional pathology in males and females. This chapter provides the first demonstration of severe diastolic dysfunction in male T1D with elevated cardiac AngII, which was not as apparent in the wildtype setting. In females, T1D induced diastolic functional detriment was similar in wildtype and AngII-Tg mice. Some evidence of sex differences were present with male-specific exacerbation of T1D-induced wall thinning, marked ischemic injury and “*loss of benefit*” in post-ischemic recovery with cardiac AngII overexpression, compared to the Wildtype setting. The lack of T1D anatomic cardiopathology in females with high AngII is suggestive of local estrogen-AngII regulation in diabetic hearts, which requires further exploration.

6.6 Limitations

One of the limitations of this study was that intracellular cardiac AngII levels were not measured in male and female diabetics, making it difficult to establish a strong association between sex steroids, local AngII levels and the associated T1D-induced changes. The cross reactivity issues between AngII, AngI and Ang inactive peptides makes them particularly difficult to measure using conventional radio immunoassays.

Isolating local cardiac AngII and measuring concentration through mass spectrometry with a synthetic AngIV analogue as an internal standard are under consideration.

6.7 References

Baker KM, and Kumar R. Intracellular angiotensin ii induces cell proliferation independent of at1 receptor. *Am J Physiol Cell Physiol* 2006 **291**: C995-1001.

Bell JR, Mellor KM, Wollermann AC, and Delbridge LM. Cardiac ischaemic stress: Cardiomyocyte ca(2)(+), sex and sex steroids. *Clin Exp Pharmacol Physiol* 2011 **38**: 717-723.

Benazzoug Y, Borchellini C, Labat-Robert J, Robert L, and Kern P. Effect of high-glucose concentrations on the expression of collagens and fibronectin by fibroblasts in culture. *Exp Gerontol* 1998 **33**: 445-455.

Boyer JK, Thanigaraj S, Schechtman KB, and Perez JE. Prevalence of ventricular diastolic dysfunction in asymptomatic, normotensive patients with diabetes mellitus. *Am J Cardiol* 2004 **93**: 870-875.

Bundalo M, Zivkovic M, Tepavcevic S, Culafic T, Koricanac G, and Stankovic A. Fructose-rich diet-induced changes in the expression of the renin angiotensin system molecules in the heart of ovariectomized female rats could be reversed by estradiol. *Horm Metab Res* 2015 **47**: 521-527.

Connelly KA, Kelly DJ, Zhang Y, Prior DL, Martin J, Cox AJ, Thai K, Feneley MP, Tsoporis J, White KE, Krum H, and Gilbert RE. Functional, structural and molecular aspects of diastolic heart failure in the diabetic (mren-2)27 rat. *Cardiovasc Res* 2007 **76**: 280-291.

Cooper SA, Whaley-Connell A, Habibi J, Wei Y, Lastra G, Manrique C, Stas S, and Sowers JR. Renin-angiotensin-aldosterone system and oxidative stress in cardiovascular insulin resistance. *Am J Physiol Heart Circ Physiol* 2007 **293**: H2009-2023.

Dalpiaz PL, Lamas AZ, Caliman IF, Ribeiro RF, Jr., Abreu GR, Moyses MR, Andrade TU, Gouvea SA, Alves MF, Carmona AK, and Bissoli NS. Sex hormones promote opposite effects on ace and ace2 activity, hypertrophy and cardiac contractility in spontaneously hypertensive rats. *PLoS One* 2015 **10**: e0127515.

Domenighetti AA, Wang Q, Egger M, Richards SM, Pedrazzini T, and Delbridge LM. Angiotensin ii-mediated phenotypic cardiomyocyte remodeling leads to age-dependent cardiac dysfunction and failure. *Hypertension* 2005 **46**: 426-432.

Dostal DE, and Baker KM. Angiotensin ii stimulation of left ventricular hypertrophy in adult rat heart. Mediation by the at1 receptor. *Am J Hypertens* 1992 **5**: 276-280.

Farah V, Elased KM, and Morris M. Genetic and dietary interactions: Role of angiotensin at1a receptors in response to a high-fructose diet. *Am J Physiol Heart Circ Physiol* 2007 **293**: H1083-1089.

Feldmer M, Kaling M, Takahashi S, Mullins JJ, and Ganten D. Glucocorticoid- and estrogen-responsive elements in the 5'-flanking region of the rat angiotensinogen gene. *J Hypertens* 1991 **9**: 1005-1012.

Frustaci A, Kajstura J, Chimenti C, Jakoniuk I, Leri A, Maseri A, Nadal-Ginard B, and Anversa P. Myocardial cell death in human diabetes. *Circ Res* 2000 **87**: 1123-1132.

Gusev K, Domenighetti AA, Delbridge LM, Pedrazzini T, Niggli E, and Egger M. Angiotensin ii-mediated adaptive and maladaptive remodeling of cardiomyocyte excitation-contraction coupling. *Circ Res* 2009 **105**: 42-50.

Harada K, Komuro I, Shiojima I, Hayashi D, Kudoh S, Mizuno T, Kijima K, Matsubara H, Sugaya T, Murakami K, and Yazaki Y. Pressure overload induces cardiac hypertrophy in angiotensin ii type 1a receptor knockout mice. *Circulation* 1998 **97**: 1952-1959.

Kamide K, Rakugi H, Higaki J, Okamura A, Nagai M, Moriguchi K, Ohishi M, Satoh N, Tuck ML, and Ogihara T. The renin-angiotensin and adrenergic nervous system in cardiac hypertrophy in fructose-fed rats. *Am J Hypertens* 2002 **15**: 66-71.

Luther JM, and Brown NJ. The renin-angiotensin-aldosterone system and glucose homeostasis. *Trends Pharmacol Sci* 2011 **32**: 734-739.

Masuda T, Muto S, Fujisawa G, Iwazu Y, Kimura M, Kobayashi T, Nonaka-Sarukawa M, Sasaki N, Watanabe Y, Shinohara M, Murakami T, Shimada K,

Kobayashi E, and Kusano E. Heart angiotensin ii-induced cardiomyocyte hypertrophy suppresses coronary angiogenesis and progresses diabetic cardiomyopathy. *Am J Physiol Heart Circ Physiol* 2012 **302**: H1871-1883.

Mazzolai L, Nussberger J, Aubert JF, Brunner DB, Gabbiani G, Brunner HR, and Pedrazzini T. Blood pressure independent cardiac hypertrophy induced by locally activated renin-angiotensin system. *Hypertension* 1998 **31**: 1324-1330.

Mazzolai L, Pedrazzini T, Nicoud F, Gabbiani G, Brunner HR, and Nussberger J. Increased cardiac angiotensin ii levels induce right and left ventricular hypertrophy in normotensive mice. *Hypertension* 2000 **35**: 985-991.

Mellor KM, Curl CL, Chandramouli C, Pedrazzini T, Wendt IR, and Delbridge LM. Ageing-related cardiomyocyte functional decline is sex and angiotensin ii dependent. *Age (Dordr)* 2014 **36**: 9630.

Porrello ER, and Delbridge LM. Cardiomyocyte autophagy is regulated by angiotensin ii type 1 and type 2 receptors. *Autophagy* 2009 **5**: 1215-1216.

Rabi DM, Khan N, Vallee M, Hladunewich MA, Tobe SW, and Pilote L. Reporting on sex-based analysis in clinical trials of angiotensin-converting enzyme inhibitor and angiotensin receptor blocker efficacy. *Can J Cardiol* 2008 **24**: 491-496.

Rodriguez R, Viscarra JA, Minas JN, Nakano D, Nishiyama A, and Ortiz RM. Angiotensin receptor blockade increases pancreatic insulin secretion and decreases glucose intolerance during glucose supplementation in a model of metabolic syndrome. *Endocrinology* 2012 **153**: 1684-1695.

Singh VP, Le B, Khode R, Baker KM, and Kumar R. Intracellular angiotensin ii production in diabetic rats is correlated with cardiomyocyte apoptosis, oxidative stress, and cardiac fibrosis. *Diabetes* 2008 **57**: 3297-3306.

Sugaya T, Nishimatsu S, Tanimoto K, Takimoto E, Yamagishi T, Imamura K, Goto S, Imaizumi K, Hisada Y, Otsuka A, and et al. Angiotensin ii type 1a receptor-deficient mice with hypotension and hyperreninemia. *J Biol Chem* 1995 **270**: 18719-18722.

Sullivan JC. Sex and the renin-angiotensin system: Inequality between the sexes in response to ras stimulation and inhibition. *Am J Physiol Regul Integr Comp Physiol* 2008 **294**: R1220-R1226.

Thomas CM, Yong QC, Seqqat R, Chandel N, Feldman DL, Baker KM, and Kumar R. Direct renin inhibition prevents cardiac dysfunction in a diabetic mouse model: Comparison with an angiotensin receptor antagonist and angiotensin-converting enzyme inhibitor. *Clin Sci (Lond)* 2013 **124**: 529-541.

Yong QC, Thomas CM, Seqqat R, Chandel N, Baker KM, and Kumar R. Angiotensin type 1a receptor-deficient mice develop diabetes-induced cardiac dysfunction, which is prevented by renin-angiotensin system inhibitors. *Cardiovasc Diabetol* 2013 **12**: 169.

Zabalgaitia M, Ismaeil MF, Anderson L, and Maklady FA. Prevalence of diastolic dysfunction in normotensive, asymptomatic patients with well-controlled type 2 diabetes mellitus. *Am J Cardiol* 2001 **87**: 320-323.

Table 6.1 Systemic and cardiac morphologic characteristics of T1D male and female AngII-Tg mice.

	AngII-Tg Male		AngII-Tg Female	
	Ctrl	STZ	Ctrl	STZ
Blood glucose (mM)	9.6 ± 0.9	26.1 ± 1.3*	8.4 ± 0.3	14.4 ± 1.8* [#]
Body weight (g)	32.3 ± 0.8	26.7 ± 0.6*	25.2 ± 1.0 [#]	22.8 ± 0.5* [#]
Tibia length (mm)	17.7 ± 0.3	17.3 ± 0.2	17.2 ± 0.2	17.2 ± 0.2
Heart weight (mg)	186 ± 8	143 ± 5*	134 ± 4 [#]	121 ± 3 [#]
Heart weight/body weight (mg/g)	5.8 ± 0.3	5.4 ± 0.2	5.4 ± 0.1	5.4 ± 0.1
Heart weight/tibia length (mg/mm)	10.5 ± 0.5	8.3 ± 0.3*	7.8 ± 0.2 [#]	7.0 ± 0.2 [#]

Data are presented as mean ± sem (n=13-17/grp). *p<0.05 vs. Ctrl, [#]p<0.05 vs. male, 2-way ANOVA, annotated with LSD post-hoc analyses. Heart weights were measured post ischemia-reperfusion.

Table 6.2 Echocardiographic function characteristics of T1D male and female AngII-Tg mice.

	Male		Female	
	Ctrl	STZ	Ctrl	STZ
E wave (mm/s)	501 ± 14	492 ± 26	441 ± 31	496 ± 24
A wave (mm/s)	322 ± 35	365 ± 25	315 ± 28	410 ± 7*
E/A ratio	1.61 ± 0.12	1.39 ± 0.13	1.45 ± 0.11	1.23 ± 0.05
E' wave (mm/s)	28.5 ± 4.0	21.3 ± 4.4	29.2 ± 2.6	26.0 ± 2.1
A' wave (mm/s)	21.9 ± 1.3	22.7 ± 2.2	24.1 ± 2.6	32.1 ± 2.4* [#]
E'/A' ratio	1.27 ± 0.13	0.96 ± 0.19	1.23 ± 0.04	0.82 ± 0.05*
PHT (ms)	10.3 ± 0.8	14.6 ± 1.9	8.8 ± 0.6	15.6 ± 2.3*
Dec Slope (mm/s ²)	14507 ± 870	10918 ± 1467	15591 ± 990	11571 ± 1699
End systolic vol. (μL)	52 ± 14	76 ± 11	44 ± 5.4	38 ± 4.6 [#]
End diastolic vol. (μL)	143 ± 20	186 ± 15*	130 ± 6.3	108 ± 8.5 [#]
Heart rate (bpm)	442 ± 16	445 ± 21	458 ± 22	487 ± 14
Stroke vol. (μL)	92 ± 7.7	112 ± 7.4*	87 ± 3.0	70 ± 5.3 [#]
Cardiac output (mL/s)	0.68 ± 0.06	0.81 ± 0.04*	0.66 ± 0.04	0.56 ± 0.04 [#]
Interventricular septum, diastole (mm)	1.06 ± 0.03	0.87 ± 0.03	1.01 ± 0.05	1.01 ± 0.07
Interventricular septum, systole (mm)	1.55 ± 0.04	1.29 ± 0.04*	1.50 ± 0.08	1.45 ± 0.09
LV internal diameter, systole (mm)	2.55 ± 0.21	3.02 ± 0.16*	2.51 ± 0.10	2.41 ± 0.09 [#]

Mitral valve flow velocity during the early ventricular filling phase (E wave) and during atrial contraction (A wave); mitral valve tissue movement velocity at the early ventricular filling phase (E' wave) and during atrial contraction (A' wave); pressure half time (PHT); deceleration slope (Dec Slope). Data are presented as mean ± sem (n=5-10/grp). *p<0.05 vs. Ctrl, [#]p<0.05 vs. male, 2-way ANOVA, annotated with LSD post-hoc analyses.

Table 6.3 Basal *ex vivo* cardiac function in T1D male and female AngII-Tg mice.

	Male		Female	
	Ctrl	STZ	Ctrl	STZ
LVEDP (mmHg)	5.33 ± 1.0	7.47 ± 0.97	5.54 ± 0.89	8.16 ± 1.4
LV DevP (mmHg)	80.9 ± 9.4	95.1 ± 13	96.4 ± 9.6	79.7 ± 8.0
dP/dt _{max} (mmHg/sec)	2740 ± 270	3430 ± 340	3350 ± 360	2380 ± 390 [#]
dP/dt _{min} (mmHg/sec)	-1910 ± 200	-2630 ± 340	-2410 ± 210	-1730 ± 240 [#]
Coronary flow (ml/min)	2.38 ± 0.33	2.65 ± 0.19	2.07 ± 0.022	2.15 ± 0.15
Heart rate (non-paced, bpm)	350 ± 29	325 ± 27	359 ± 14	357 ± 10

Left ventricular end diastolic pressure (LV EDP), left ventricular developed pressure (LV DevP), left ventricular maximum rate of contraction (dP/dt_{max}), left ventricular maximum rate of relaxation (dP/dt_{min}). Data are presented as mean ± sem (n=6-7/grp).

*p<0.05 *vs* Ctrl, [#]p<0.05 *vs* male, 2-way ANOVA, annotated with LSD post-hoc analyses.

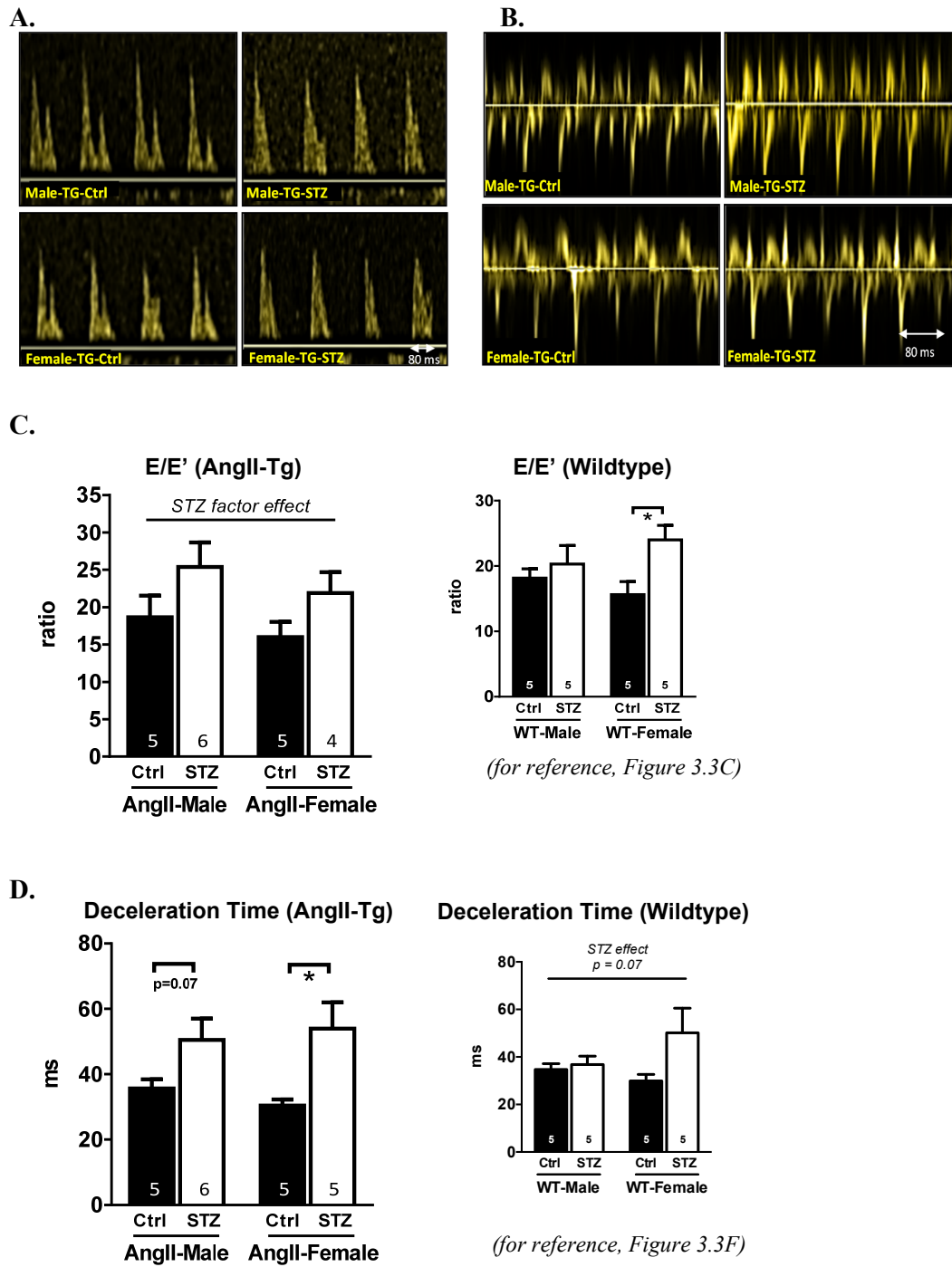


Figure 6.1. Diastolic dysfunction is evident in male and female T1D mice with elevated cardiac AngII. **A.** Representative traces from A4C view of pulse-wave (blood flow) Doppler; **B.** Representative traces from A4C view of mitral valve tissue Doppler. **C.** E/E' ratio. **D.** Mitral valve deceleration time. Data are presented as mean $\pm$ sem. * $p < 0.05$, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

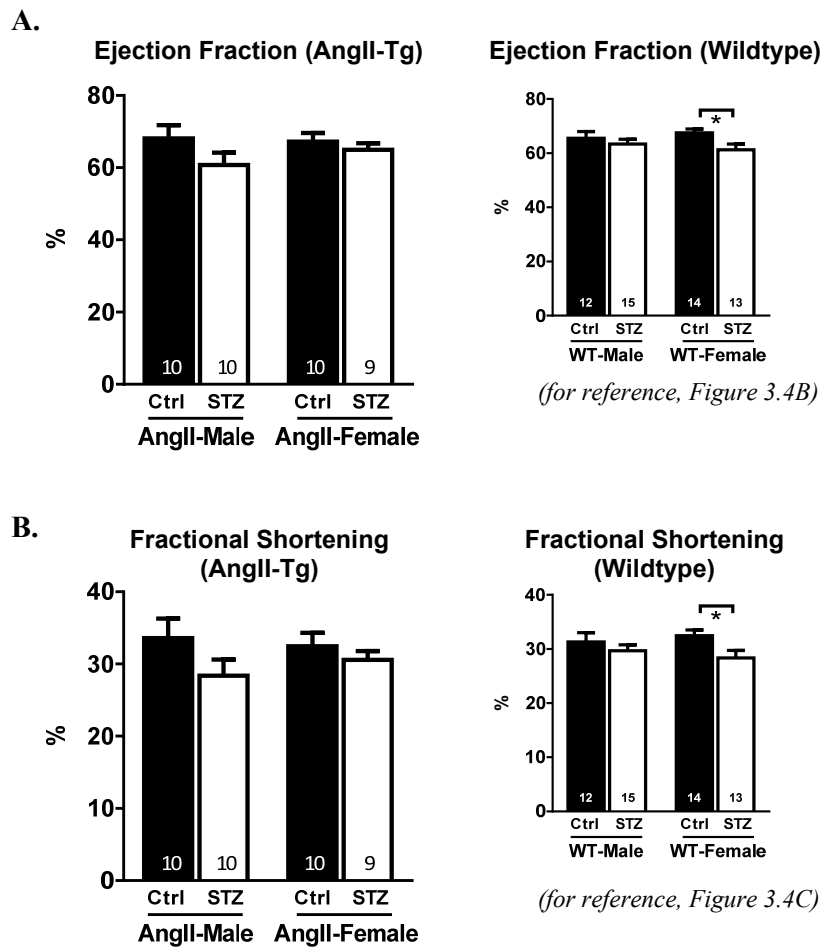
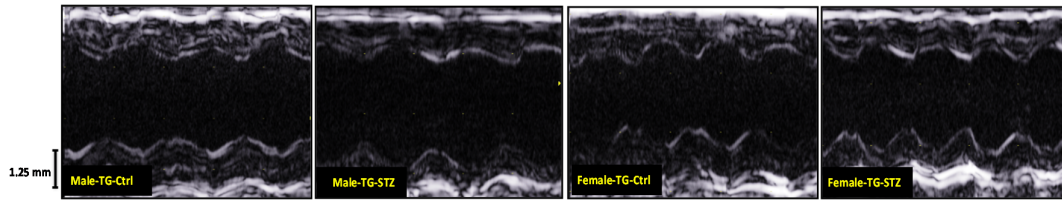
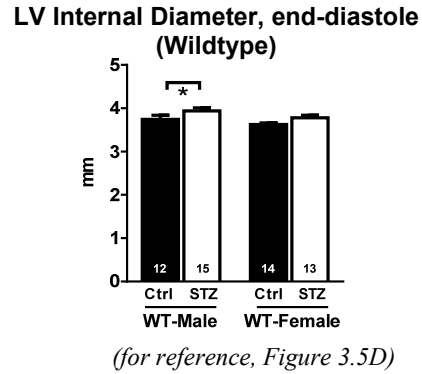
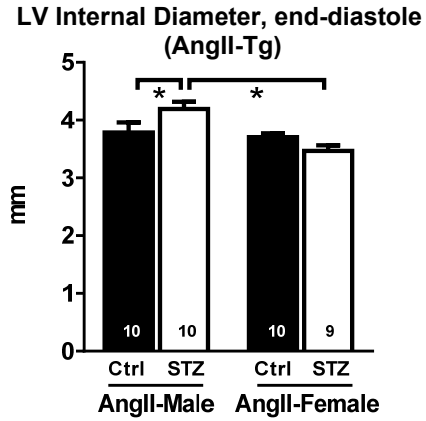


Figure 6.2. Systolic dysfunction is not evident in male and female T1D mice with elevated cardiac AngII. A. Ejection fraction. **B.** Fractional shortening. Data are presented as mean $\pm$ sem. * $p < 0.05$, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

A.



B.



C.

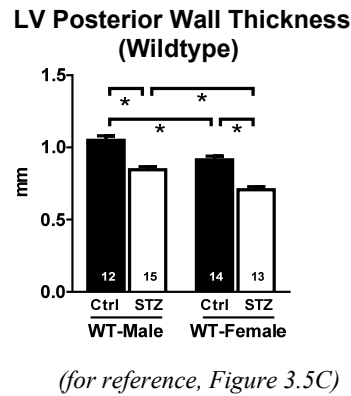
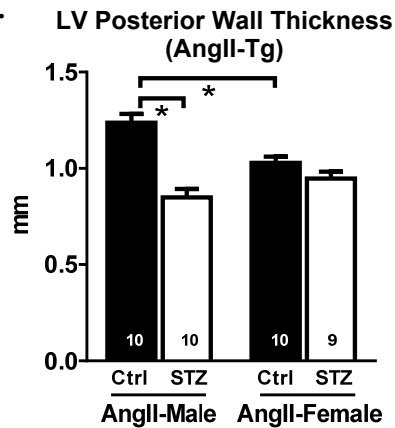


Figure 6.3. Left ventricular dilation and wall thinning is evident in male but not female T1D mice with elevated cardiac AngII. A. Representative m-mode images of LV short axis view. **B.** LV internal diameter at diastole. **C.** Posterior wall thickness at diastole. Data are presented as mean $\pm$ sem. * $p < 0.05$, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

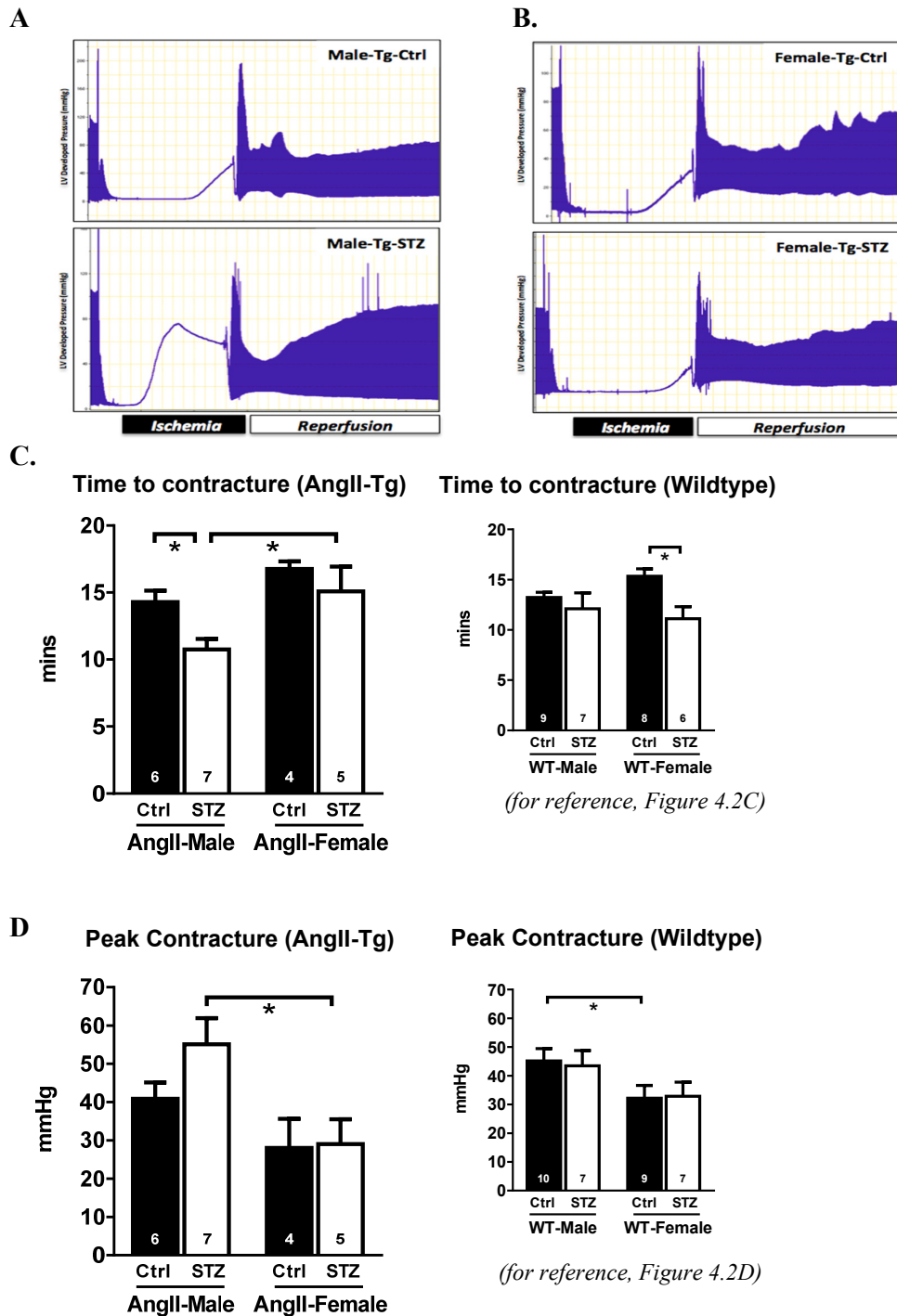


Figure 6.4. Female ischemic injury in T1D mice with elevated cardiac AngII is less severe than in male mice. **A.** Representative pressure traces of AngII-Tg male control and STZ mouse hearts throughout the ischemia-reperfusion protocol. **B.** Representative pressure traces of AngII-Tg female control and STZ mouse hearts throughout the ischemia-reperfusion protocol. **C.** Time to onset of ischemic contracture. **D.** Amplitude of ischemic contracture. Data are presented as mean $\pm$ sem. * $p < 0.05$, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

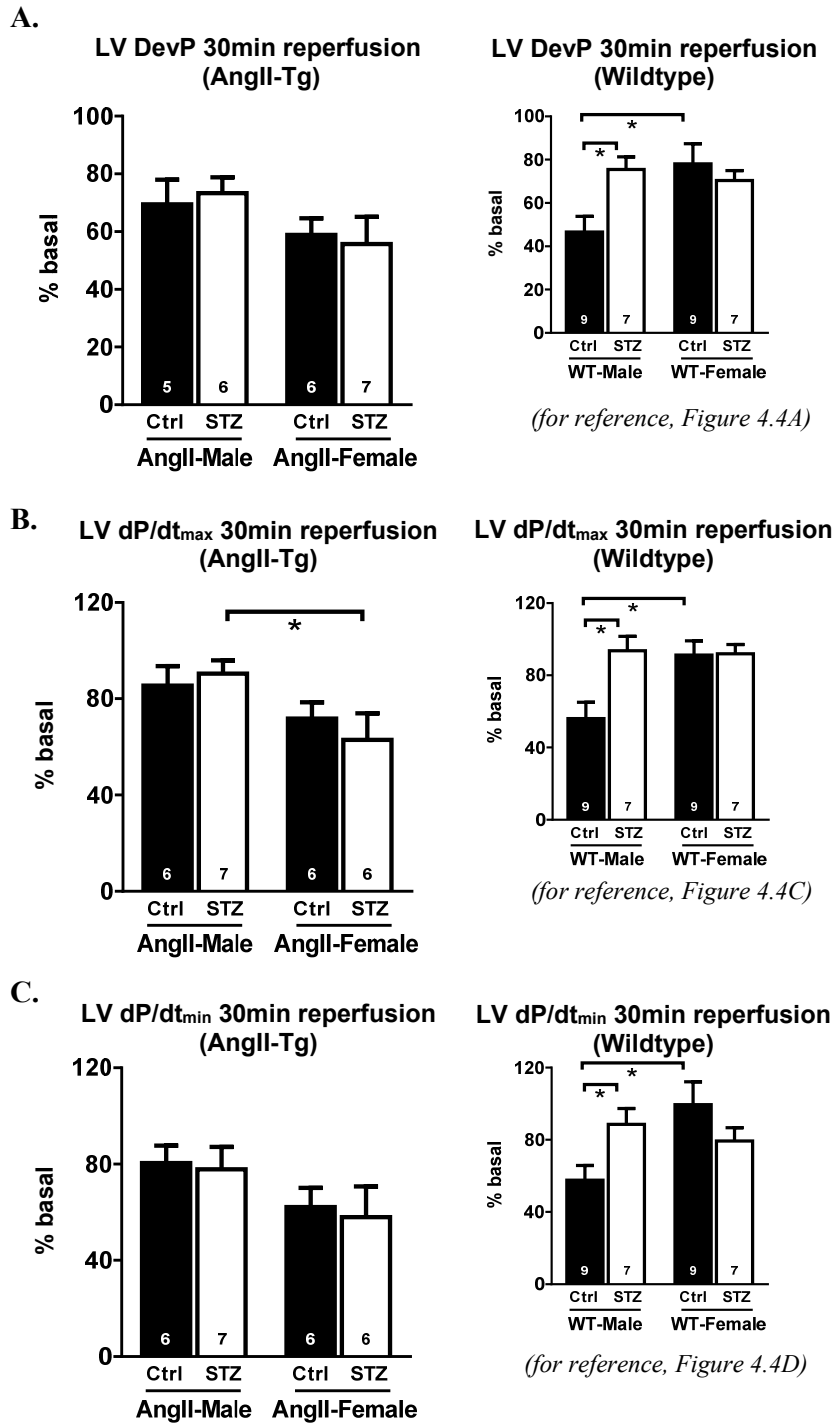


Figure 6.5. No effect of T1D on contractile recovery post-ischemia in male and female mice with elevated cardiac AngII. **A.** Percentage recovery of systolic function (left ventricular developed pressure, LV DevP). **B.** Percentage recovery of left ventricular maximum rate of contraction (dP/dt_{max}). **C.** Percentage recovery of left ventricular maximum rate of relaxation (dP/dt_{min}). Data are presented as mean ± sem. *p<0.05, 2-way ANOVA, annotated with LSD post-hoc analyses. Sample size (n/group) indicated within bars of graphs.

CHAPTER 7

General discussion and conclusions

7.1 Overview

Diabetes is a disease of epidemic proportions with significant economic burden on countries worldwide (Boudina *et al.*, 2007). Diabetic cardiomyopathy is a distinct cardiopathology, independent of other risk factors, e.g. hypertension and coronary artery disease. Accumulating clinical evidence has identified that cardiovascular consequences of diabetes in women are particularly dire. The Framingham Heart Study was the pioneer in identifying a 5-fold increase in cardiovascular risk among diabetic women compared to a 2-fold increase in risk in males (Kannel *et al.*, 1979). Diabetic females are characterised by higher risk of acute myocardial infarction than their male diabetic counterparts (Kappert *et al.*, 2012; Lam *et al.*, 2012). Worsened post-infarction outcomes for diabetic women have been documented in studies of large cohorts, conducted in globally diverse locations and including women of varying ages (Crowley *et al.*, 2003; Fraser *et al.*, 1992; Hayes *et al.*, 2011; Lundberg *et al.*, 1997; Norhammar *et al.*, 2008; Seeman *et al.*, 1993).

Since Framingham, considerable progress has been made in developing experimental models of diabetic disease states, including a plethora of genetic rodent diabetic models utilised to understand the molecular cardiopathology. The cardiac functional deficit consistently observed involves an underlying diastolic dysfunction preceding systolic abnormality (Boyer *et al.*, 2004; Zabalgaitia *et al.*, 2001). Structural diabetic pathology manifests as loss of myocardial collagen elasticity and cardiomyocyte loss (Regan *et al.*, 1977; Shimizu *et al.*, 1993). Metabolic perturbations include a shift towards higher reliance on fatty acid in the insulin-deficient myocardium (Han *et al.*, 2014; Liu *et al.*, 2001; Pham *et al.*, 2014; van Heerebeek *et al.*, 2008). Perplexingly, little experimental work has explored sex-specific outcomes in diabetic cardiomyopathy. The case for mechanistic interrogation of sex differences, of sex steroid influences in the aetiology of diabetic cardiomyopathy, is particularly compelling.

The experiments undertaken in this thesis were designed to examine female susceptibility to cardiac pathology in type 1 diabetes (T1D), with a particular focus on cardiac function (*in vivo* and *ex vivo*), vulnerability to ischemic stress (*ex vivo*), cellular metabolism (molecular) and involvement from the renin-angiotensin system (*in vivo* and *ex vivo*). The novel finding that less pronounced hyperglycaemic insult

was linked with more severe diastolic dysfunction in female T1D (compared to males) shaped the direction of this thesis to further explore the mechanistic basis of female cardiac vulnerability in T1D.

The four experimental questions addressed in this thesis are:

1. Are systemic and cardiac diabetic phenotypes different between males and females? [Chapter 3]
2. Is there an accentuated female vulnerability to ischemia reperfusion injury in diabetes? [Chapter 4]
3. Are there sex-specific changes in metabolism and cell viability with diabetes? [Chapter 5]
4. Does cardiac RAS upregulation interact with sex-specific cardiac responses in diabetes? [Chapter 6]

The major findings of these investigations are that:

1. Despite less pronounced systemic increases in diabetic diagnostic markers (in female T1D mice comparison to male mice with T1D), diastolic dysfunction was evident in female T1D but not in males (Figure 3.3C).
2. In males, diabetic hearts showed greater functional rebound post-ischemia during acute reperfusion which was associated with reduced cardiac glycogen levels, suggesting better glycogen utilisation during ischemia. In contrast, female hearts exhibited an indication of greater injury (Figure 4.2C) with unchanged glycogen levels with T1D.
3. Genes responsible for lysosomal breakdown of glycogen (glycophagy) were upregulated in T1D males, whereas, genes from cytosolic enzyme-regulated glycogen breakdown were increased in female T1D (Figure 5.1A). A marked increase in expression of genes relating to macrophagy (autophagic protein bulk degradation) and apoptotic cell death was observed in female but, not in male T1D (Figure 5.1A).
4. With elevated cardiac AngII, T1D-induced cardiac functional and structural changes were exacerbated in males but not as apparent in females.

The importance of these findings is discussed in the following sections of this chapter.

7.2 Evidence of distinct systemic and cardiac diabetic pathology in both sexes

More than three decades ago, the Framingham Heart Study identified an accentuated female vulnerability in diabetic cardiomyopathy (Kannel *et al.*, 1979). Since then, clinical studies have repeatedly confirmed this “*disadvantage*” in diabetic females (Barrett-Connor *et al.*, 1991; Folsom *et al.*, 1997; Heyden *et al.*, 1980; Natarajan *et al.*, 2003; Simons *et al.*, 1996). Despite the case for an earlier onset of diastolic dysfunction with T1D in women, potential sex-specific investigations utilising experimental T1D models have been minimal in number (Reichelt *et al.*, 2013).

Elevated fasting glucose is the primary diabetic diagnostic, and may be used as the sole criterion in determining diabetes prevalence and incidence, as recommended by the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (ECDCDM, 2003). The research findings reported in Chapter 3 of this thesis are the first to demonstrate that a less pronounced increase in blood glucose, HbA1c and glucose intolerance were linked with more severe diastolic dysfunction in T1D females. Systolic function remained preserved with T1D in both sexes. Similar extent of fibrosis and wall thinning were observed with T1D in both sexes. Molecular evaluations suggest selective upregulation of genes involved in glycolysis and glycogen enzymatic breakdown in female, but not male, hearts with diabetes.

These findings are the first to report that a less pronounced systemic insult, ie. more modest hyperglycaemia, is linked to more severe cardiopathology in female T1D. A lower diabetic diagnostic threshold could be considered for females, as glycemic status may not reflect the underlying cardiac pathology. The current usage of similar glycemic cut-offs for both sexes may not be sufficient in diagnosing early stages of diabetic cardiomyopathy in women. Thus, the adoption of sex-specific cut-offs seems appropriate for earlier diagnosis and more effective management of cardiac pathology in diabetic females. Whether the same will be applicable to T2D and human diabetics is pending confirmation. In addition, heart failure in STZ-induced T1D mice manifests as pronounced diastolic dysfunction, with preserved ejection fraction. Beyond diuretics, there are no specific treatment options tailored for this condition as yet. More research is warranted to examine molecular targets of diastolic diabetic

cardiomyopathy. The systemic, cardiac structural and functional relationship presented in Chapter 3 has provided a better understanding of diabetic heart failure with preserved ejection fraction (HFpEF) in both sexes.

7.3 Female vulnerability in ischemia reperfusion linked with diminished glycogen utilisation

Clinically, increased susceptibility to ischemic heart disease among diabetic patients has been documented (IDF, 2015). Poorer prognosis following an infarct event has been reported in diabetic women more than in men (Crowley A *et al.*, 2003; Fraser GE *et al.*, 1992). Limited sex-specific studies and the varying extent of ischemic susceptibility in experimental models have made it difficult to fully understand ischemic pathology among diabetics.

Chapter 4 of this thesis examined whether these sex differences in metabolic and functional adaptations in T1D are more apparent following ischemia reperfusion, which conferred an additional nutrient challenge to the diabetic myocardium. Using an isolated heart model, ischemic damage and functional rebound following an acute reperfusion period were assessed *ex vivo* in T1D male and female hearts. Tissue analysis was performed at the end of reperfusion to measure cardiac glycogen content. The novel sex-specific findings from this chapter show that male T1D mice exhibited greater recovery in acute reperfusion and were associated with reduced cardiac glycogen stores at the end of reperfusion. Despite the earlier onset of ischemic contracture in T1D females, subsequent functional recovery at the end of acute reperfusion was unchanged. Interestingly, female T1D hearts also had higher free glucose stores and unaltered glycogen content, suggesting diminished utilisation to glycogen stores. These novel findings suggest sex-specific glycolytic fuel management during ischemic reperfusion, which may be important in determining the extent of ischemic injury in the heart. While the functional rebound observed, particularly in male T1D, did not align with clinical reports, various *ex vivo* experimental studies have reported the same (Ma *et al.*, 2006; Nawata *et al.*, 2002; Ooie *et al.*, 2003; Shi-ting *et al.*, 2010; Tani *et al.*, 1988). This discrepancy can be attributed to (1) the availability of glucose as the only substrate in the perfusion buffer, (2) functional assessment being conducted following an acute, not chronic, reperfusion setting and (3) a less severe diabetic phenotype.

These findings have added important sex-specific insights in the occurrence of acute myocardial infarction and risk of developing left ventricular dysfunction post infarct. The greater indication of ischemic injury and diminished access to glycolytic fuel in female diabetic mice presented in this chapter could potentially be extended to diabetic women with increased vulnerability to heart failure post infarct. These findings warrant a more extensive molecular investigation of key metabolic and cell viability, which may be altered in both sexes, contributing to the differential functional and ischemic pathology.

7.4 Differential expression of glycogen handling, autophagy and apoptosis genes in diabetic male and female hearts

Cardiac-specific metabolic alterations in diabetes are evident and have been associated with cell death (Han *et al.*, 2014; Liu *et al.*, 2001; Mellor *et al.*, 2011a; Pham *et al.*, 2014; van Heerebeek *et al.*, 2008). Perplexingly, glycogen accumulation occurs in diabetic hearts despite reduced glucose uptake with an insulin deficient or resistant state (Alfarano *et al.*, 2011; Lajoie *et al.*, 2004; Mowry *et al.*, 1951; Sakakibara *et al.*, 2011; Shearer *et al.*, 2011; Warren, 1930). In addition to apoptotic cell loss, evidence of disturbed phagic processing of macromolecules in diabetic hearts has emerged in recent years (Delbridge *et al.*, 2015; Kobayashi *et al.*, 2012; Mellor *et al.*, 2011b; Wang *et al.*, 2013; Xie, 2012; Xie *et al.*, 2011; Zou *et al.*, 2013). The utilisation of various experimental diabetic rodent models with diverse metabolic perturbation profiles poses an ongoing challenge in understanding the underlying molecular pathology of diabetes. Sex-specific differences have not been explored. Chapter 5 of this thesis provides a comprehensive report of gene expression changes in key metabolic, signalling, macroautophagy and glycophyagy pathways in diabetic males and females. Directional changes in gene expression in males and females are summarised in Table 7.1 below.

A female-specific increase in both myocardial autophagic and apoptotic cell death was evident with chronic T1D, which was absent in males, pending confirmation with protein assessment. Additionally, preliminary finding of glycogen breakdown through cytosolic enzyme-regulated process was present in female diabetics while bulk glycophyagy breakdown of glycogen was occurring in male diabetics is also reported. The “*quicker*” and efficient access to glucose in male diabetic hearts possibly blunted

the increase in energy stress causing no major perturbations in cell death pathways and assisted in preserving cardiac function to some extent. In contrast, the insulin-deficient female hearts increased autophagic drive, perhaps to compensate for inefficiency in glycogen breakdown. This excessive macroautophagy drive perhaps turned maladaptive, as it was also associated with increased expression of apoptotic genes, a significant structural and functional deficit with diabetes in females. This novel observation of female-specific upregulation in autophagy response in the T1D heart could be pivotal in sex-specific therapeutic targets from phagic pathways.

This chapter has also extended findings from the chronic T1D model to other rodent diabetic models of T2D. Interestingly, contrasting responses were found in lean and obese T2D models. Lean T2D hearts with insulin resistance displayed downregulated insulin signalling and increased autophagy. In addition to insulin resistance, the obesity phenotype in obese T2D resulted in a suppression of autophagy, possibly due to high intracellular nutrient energy status. Interestingly, both increased and decreased autophagy was associated with impaired cardiac function in lean (Mellor *et al.*, 2011a) and obese T2D (Huynh *et al.*, 2012) respectively. Autophagy in diabetes appears to follow the “*Goldilocks*” phenomenon, too little or too much can both result in detrimental consequences. This is a key finding for basic science and translational studies focusing on autophagic signalling pathways for therapeutic potential. Experimental investigations solely aiming to either suppress or increase autophagy may not be as effective and should not be ubiquitously applied to all diabetic conditions. These findings provide important leads for future investigations and determining whether these gene expression changes are compensatory or causative is a key priority.

Table 7.1: Summary of gene expression changes with T1D in males and female mice

Signalling pathway	Male STZ	Female STZ	Lean T2D	Obese T2D
Glycogen enzymatic breakdown	=	↑	↑	↓
Glycogen glycopphagic breakdown	↑	=	↑	↑
Macrophagy	=	↑	↑	↓
Apoptosis	=	↑	=	↓

7.5 T1D-induced cardiac functional and structural detriments are exacerbated by elevated cardiac AngII in male but not female mice.

Left ventricular dysfunction in diabetic cardiomyopathy is often associated with increased activation of the renin-angiotensin (AngII) system. Inhibition of cardiac AngII results in improved systolic and diastolic function in diabetic rodents (Masuda *et al.*, 2012). RAS inhibitors have shown greater efficacy in male compared to female patients (Rabi *et al.*, 2008). The role of sex differences in the pathogenesis of diabetic cardiomyopathy in elevated cardiac AngII setting was yet to be investigated.

Chapter 6 of this thesis utilised a cardiac-specific AngII overexpressing transgenic mouse model to investigate sex differences in T1D mediated deterioration of function (*in vivo*) and acute ischemia reperfusion (*ex vivo*). Findings reported in this chapter provide the first demonstration of severe diastolic dysfunction in male T1D with elevated cardiac AngII, which was not as apparent in the wildtype setting. In females, T1D-induced diastolic functional detriment was similar in wildtype and AngII-Tg mice. Some evidence of sex differences was present with male-specific exacerbation of T1D-induced wall thinning, marked ischemic injury and “*loss of benefit*” in post-ischemic recovery with cardiac AngII overexpression, compared to the wildtype setting. The lack of T1D anatomic cardiopathology in females with high AngII is suggestive of local estrogen-AngII regulation in diabetic hearts, which requires further exploration.

These findings presented in Chapter 6 were surprising but informative of sex-specific differences in the diabetic heart when cardiac AngII levels are elevated. These findings provide insight possible local interaction of estrogen and cardiac AngII, which may translate into differential effects of administration of RAS inhibitors in females. Specifically, two main questions to be addressed to achieve a better understanding on this interaction are: 1) Do sex differences in metabolic fuel handling in diabetes become more apparent in an elevated AngII setting? 2) Is there a role for local cardiac AngII in increased autophagic drive in female diabetics? Experimental pursuits to unravel the complex relationship between estrogen, AngII and insulin signalling pathway are warranted.

7.6 New research questions

The findings reported in this thesis have inspired new research questions in the field of sex differences in diabetic cardiomyopathy. The novel finding that a less pronounced systemic insult was linked with worse diastolic functional pathology provides momentum to investigate sex differences in glucose handling among diabetics using animal models. The possibility that basal glucose uptake, independent of insulin, could perhaps be greater in female diabetics resulting in a lower blood glucose presentation is yet to be investigated in both rodents and humans.

This thesis has identified an indication of greater ischemic injury linked with diminished glycogen utilisation in female diabetic mice, but not males, following an acute reperfusion period. The clinical jeopardy in myocardial infarction among diabetic patients was not observed in this study, as in many other rodent studies on ischemia. These clinical and experimental differences are possibly attributable to *ex vivo* ischemic conditions and functional measures taken following an acute, not chronic, reperfusion period. Future studies are warranted to investigate whether an *in vivo* infarct and a prolonged reperfusion period, where the predominant diabetic fuel substrate source, i.e. fatty acids, is available, will be able to mimic the clinical responses more closely.

Several gene expression changes were highlighted in this study. Importantly, in female diabetic mice, enzyme-regulated glycogen breakdown, macrophagy and apoptotic cell death pathways were upregulated. Studies are required to confirm these gene expression changes at protein level to further pursue these mechanistic leads highlighted in glycogen breakdown and macrophagy pathways as sex-specific therapeutic targets of diabetic cardiomyopathy.

With elevated AngII, diabetes-induced functional deficit was evident in both sexes while wall-thinning pathology was exacerbated in male diabetic mice, but not in females, in this study. Given the estrogenic link with RAS components, further sex-specific exploration of local AngII modulation, myocyte viability, extent of fibrosis and glycogen fuel utilisation in diabetes are required.

7.7 Overall conclusions

Collectively, the novel findings in this thesis have contributed new knowledge to the literature on sex-specific attributes of diabetic cardiomyopathy. Key findings of this thesis are summarised in Figure 7.1. This thesis is the first to demonstrate that a less pronounced systemic insult of T1D can be associated with significant cardiac pathology in female diabetic mice. This female vulnerability may be partially attributed to a preferential slower or inefficient-processing route of glycogen and higher underlying cell death pathology, as evidenced from the pronounced autophagic drive evident in female T1D. A sex-specific role for cardiac RAS in exacerbating the T1D phenotype has also been identified. The findings reported in this thesis support the contention of an accentuated female susceptibility in diabetic cardiomyopathy and have provided new knowledge on the underlying mechanisms, enabling new sex-specific therapeutic leads to be investigated.

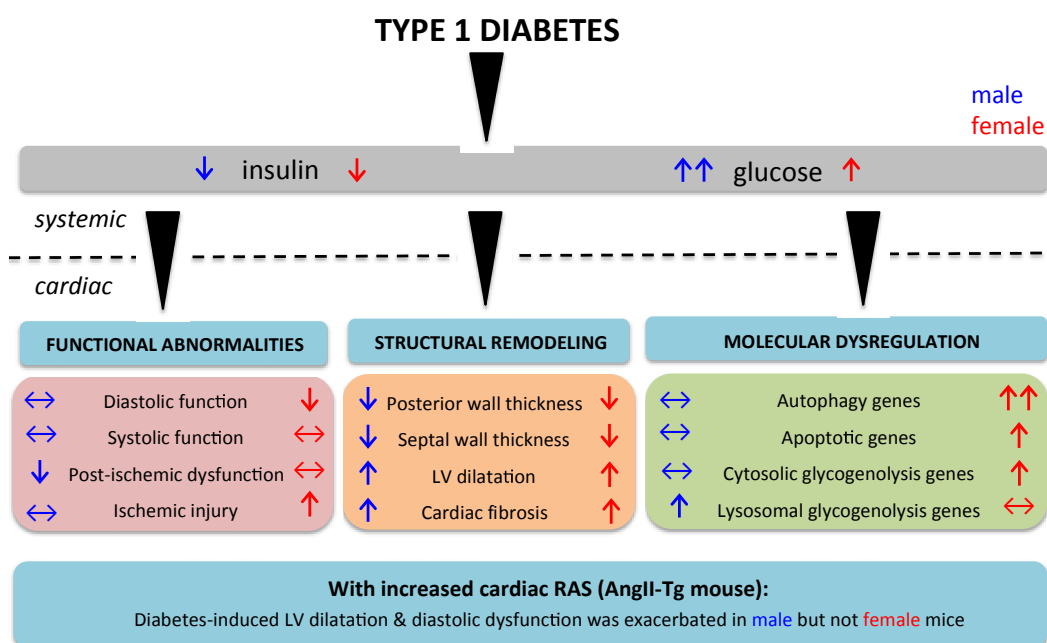


Figure 7.1: Summary of key thesis findings on sex differences in functional, structural and molecular remodelling in diabetic cardiomyopathy. Changes in each cardiac variable are indicated in blue and red arrows for males and females respectively.

7.8 References

Alfarano C, Suffredini S, Fantappie O, Mugelli A, Cerbai E, Manni ME, and Raimondi L. The effect of losartan treatment on the response of diabetic cardiomyocytes to atp depletion. *Pharmacol Res* 2011 **63**: 225-232.

Barrett-Connor EL, Cohn BA, Wingard DL, and Edelstein SL. Why is diabetes mellitus a stronger risk factor for fatal ischemic heart disease in women than in men? The rancho bernardo study. *JAMA* 1991 **265**: 627-631.

Boudina S, and Abel ED. Diabetic cardiomyopathy revisited. *Circulation* 2007 **115**: 3213-3223.

Boyer JK, Thanigaraj S, Schechtman KB, and Perez JE. Prevalence of ventricular diastolic dysfunction in asymptomatic, normotensive patients with diabetes mellitus. *Am J Cardiol* 2004 **93**: 870-875.

Crowley A, Menon V, Lessard D, Yarzebski J, Jackson E, Gore JM, and Goldberg RJ. Sex differences in survival after acute myocardial infarction in patients with diabetes mellitus (worchester heart attack study). *American heart journal* 2003 **146**: 824-831.

Delbridge LMD, Mellor KM, Taylor DJR, and Gottlieb RA. Myocardial autophagic energy stress responses—macroautophagy, mitophagy, and glycophagy. *American Journal of Physiology - Heart and Circulatory Physiology* 2015 **308**: H1194-H1204.

ECDCDM. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 2003 **26 Suppl 1**: S5-20.

Folsom AR, Szklo M, Stevens J, Liao F, Smith R, and Eckfeldt JH. A prospective study of coronary heart disease in relation to fasting insulin, glucose, and diabetes. The atherosclerosis risk in communities (aric) study. *Diabetes Care* 1997 **20**: 935-942.

Fraser GE, Strahan TM, Sabate J, Beeson WL, and Kissinger D. Effects of traditional coronary risk factors on rates of incident coronary events in a low-risk population. The adventist health study. *Circulation* 1992 **86**: 406-413.

Han JC, Goo S, Barrett CJ, Mellor KM, Taberner AJ, and Loiselle DS. The afterload-dependent peak efficiency of the isolated working rat heart is unaffected by streptozotocin-induced diabetes. *Cardiovasc Diabetol* 2014 **13**: 4.

Hayes AJ, Leal J, Kelman CW, and Clarke PM. Risk equations to predict life expectancy of people with type 2 diabetes mellitus following major complications: A study from western australia. *Diabet Med* 2011 **28**: 428-435.

Heyden S, Heiss G, Bartel AG, and Hames CG. Sex differences in coronary mortality among diabetics in evans county, georgia. *J Chronic Dis* 1980 **33**: 265-273.

Huynh K, Kiriazis H, Du XJ, Love JE, Jandeleit-Dahm KA, Forbes JM, McMullen JR, and Ritchie RH. Coenzyme q10 attenuates diastolic dysfunction, cardiomyocyte hypertrophy and cardiac fibrosis in the db/db mouse model of type 2 diabetes. *Diabetologia* 2012 **55**: 1544-1553.

IDF. Idf diabetes atlas. Brussels, Belgium: International Diabetes Federation, 2015.

Kannel WB, and McGee DL. Diabetes and cardiovascular disease. The framingham study. *Jama* 1979 **241**: 2035-2038.

Kappert K, Bohm M, Schmieder R, Schumacher H, Teo K, Yusuf S, Sleight P, and Unger T. Impact of sex on cardiovascular outcome in patients at high cardiovascular risk: Analysis of the telmisartan randomized assessment study in ace-intolerant subjects with cardiovascular disease (transcend) and the ongoing telmisartan alone and in combination with ramipril global end point trial (ontarget). *Circulation* 2012 **126**: 934-941.

Kobayashi S, Xu X, Chen K, and Liang Q. Suppression of autophagy is protective in high glucose-induced cardiomyocyte injury. *Autophagy* 2012 **8**: 577-592.

Lajoie C, Calderone A, Trudeau F, Lavoie N, Massicotte G, Gagnon S, and Beliveau L. Exercise training attenuated the pkb and gsk-3 dephosphorylation in the myocardium of zdf rats. *J Appl Physiol* 2004 **96**: 1606-1612.

Lam CS, and Little WC. Sex and cardiovascular risk: Are women advantaged or men disadvantaged? *Circulation* 2012 **126**: 913-915.

Liu JE, Palmieri V, Roman MJ, Bella JN, Fabsitz R, Howard BV, Welty TK, Lee ET, and Devereux RB. The impact of diabetes on left ventricular filling pattern in normotensive and hypertensive adults: The strong heart study. *J Am Coll Cardiol* 2001 **37**: 1943-1949.

Lundberg V, Stegmayr B, Asplund K, Eliasson M, and Huhtasaari F. Diabetes as a risk factor for myocardial infarction: Population and gender perspectives. *J Intern Med* 1997 **241**: 485-492.

Ma G, Al-Shabrawey M, Johnson JA, Datar R, Tawfik HE, Guo D, Caldwell RB, and Caldwell RW. Protection against myocardial ischemia/reperfusion injury by short-term diabetes: Enhancement of vegf formation, capillary density, and activation of cell survival signaling. *Naunyn Schmiedebergs Arch Pharmacol* 2006 **373**: 415-427.

Mellor KM, Bell JR, Young MJ, Ritchie RH, and Delbridge LM. Myocardial autophagy activation and suppressed survival signaling is associated with insulin resistance in fructose-fed mice. *J Mol Cell Cardiol* 2011a **50**: 1035-1043.

Mellor KM, Reichelt ME, and Delbridge LM. Autophagy anomalies in the diabetic myocardium. *Autophagy* 2011b **7**: 1263-1267.

Mowry RW, and Bangle R, Jr. Histochemically demonstrable glycogen in the human heart, with special reference to glycogen storage disease and diabetes mellitus. *Am J Pathol* 1951 **27**: 611-625.

Natarajan S, Liao Y, Cao G, Lipsitz SR, and McGee DL. Sex differences in risk for coronary heart disease mortality associated with diabetes and established coronary heart disease. *Arch Intern Med* 2003 **163**: 1735-1740.

Nawata T, Takahashi N, Ooie T, Kaneda K, Saikawa T, and Sakata T. Cardioprotection by streptozotocin-induced diabetes and insulin against ischemia/reperfusion injury in rats. *J Cardiovasc Pharmacol* 2002 **40**: 491-500.

Norhammar A, Stenestrand U, Lindback J, Wallentin L, Register of I, and Knowledge about Swedish Heart Intensive Care A. Women younger than 65 years with diabetes mellitus are a high-risk group after myocardial infarction: A report from

the swedish register of information and knowledge about swedish heart intensive care admission (riks-hia). *Heart* 2008 **94**: 1565-1570.

Ooie T, Takahashi N, Nawata T, Arikawa M, Yamanaka K, Kajimoto M, Shinohara T, Shigematsu S, Hara M, Yoshimatsu H, and Saikawa T. Ischemia-induced translocation of protein kinase c-epsilon mediates cardioprotection in the streptozotocin-induced diabetic rat. *Circ J* 2003 **67**: 955-961.

Pham T, Loiselle D, Power A, and Hickey AJ. Mitochondrial inefficiencies and anoxic atp hydrolysis capacities in diabetic rat heart. *Am J Physiol Cell Physiol* 2014 **307**: C499-507.

Rabi DM, Khan N, Vallee M, Hladunewich MA, Tobe SW, and Pilote L. Reporting on sex-based analysis in clinical trials of angiotensin-converting enzyme inhibitor and angiotensin receptor blocker efficacy. *Can J Cardiol* 2008 **24**: 491-496.

Regan TJ, Lyons MM, Ahmed SS, Levinson GE, Oldewurtel HA, Ahmad MR, and Haider B. Evidence for cardiomyopathy in familial diabetes mellitus. *The Journal of clinical investigation* 1977 **60**: 884-899.

Reichelt ME, Mellor KM, Bell JR, Chandramouli C, Headrick JP, and Delbridge LM. Sex, sex steroids, and diabetic cardiomyopathy: Making the case for experimental focus. *Am J Physiol Heart Circ Physiol* 2013 **305**: H779-792.

Sakakibara M, Hirashiki A, Cheng XW, Bando Y, Ohshima K, Okumura T, Funahashi H, Ohshima S, and Murohara T. Association of diabetes mellitus with myocardial collagen accumulation and relaxation impairment in patients with dilated cardiomyopathy. *Diabetes Res Clin Pract* 2011 **92**: 348-355.

Seeman T, Mendes de Leon C, Berkman L, and Ostfeld A. Risk factors for coronary heart disease among older men and women: A prospective study of community-dwelling elderly. *Am J Epidemiol* 1993 **138**: 1037-1049.

Shearer J, Ross KD, Hughey CC, Johnsen VL, Hittel DS, and Severson DL. Exercise training does not correct abnormal cardiac glycogen accumulation in the db/db mouse model of type 2 diabetes. *Am J Physiol Endocrinol Metab* 2011 **301**: E31-39.

Shi-ting W, Mang-hua X, Wen-ting C, Feng-hou G, and Zhu-ying G. Study on tolerance to ischemia-reperfusion injury and protection of ischemic preconditioning of type 1 diabetes rat heart. *Biomed Pharmacol* 2010

Shimizu M, Umeda K, Sugihara N, Yoshio H, Ino H, Takeda R, Okada Y, and Nakanishi I. Collagen remodelling in myocardia of patients with diabetes. *Journal of clinical pathology* 1993 **46**: 32-36.

Simons LA, McCallum J, Friedlander Y, and Simons J. Diabetes, mortality and coronary heart disease in the prospective dubbo study of australian elderly. *Aust N Z J Med* 1996 **26**: 66-74.

Tani M, and Neely JR. Hearts from diabetic rats are more resistant to in vitro ischemia: Possible role of altered Ca^{2+} metabolism. *Circ Res* 1988 **62**: 931-940.

van Heerebeek L, Hamdani N, Handoko ML, Falcao-Pires I, Musters RJ, Kupreishvili K, Ijsselmuiden AJ, Schalkwijk CG, Bronzwaer JG, Diamant M, Borbely A, van der Velden J, Stienen GJ, Laarman GJ, Niessen HW, and Paulus WJ. Diastolic stiffness of the failing diabetic heart: Importance of fibrosis, advanced glycation end products, and myocyte resting tension. *Circulation* 2008 **117**: 43-51.

Wang Y, Li YB, Yin JJ, Wang Y, Zhu LB, Xie GY, and Pan SH. Autophagy regulates inflammation following oxidative injury in diabetes. *Autophagy* 2013 **9**: 272-277.

Warren S. The effect of insulin on pathologic glycogen deposits in diabetes mellitus. *Am J Med Sci* 1930 **179** pp. 482–488.

Xie Z. Cardiac autophagy in diabetic cardiomyopathy. *Bioenergetics: Open access* 2012 **01**:

Xie Z, He C, and Zou MH. Amp-activated protein kinase modulates cardiac autophagy in diabetic cardiomyopathy. *Autophagy* 2011 **7**: 1254-1255.

Zabalgoitia M, Ismaeil MF, Anderson L, and Maklady FA. Prevalence of diastolic dysfunction in normotensive, asymptomatic patients with well-controlled type 2 diabetes mellitus. *Am J Cardiol* 2001 **87**: 320-323.

Zou MH, and Xie Z. Regulation of interplay between autophagy and apoptosis in the diabetic heart: New role of ampk. *Autophagy* 2013 **9**: 624-625.

CHAPTER 8

Appendix

Manuscripts derived from this chapter:

**Chandramouli C, Stevens EM, Ma B, Stapleton DS, Mellor KM, Delbridge LMD.
Measuring cardiac glycogen content. *AJP Heart Circ Phys* [In preparation]**

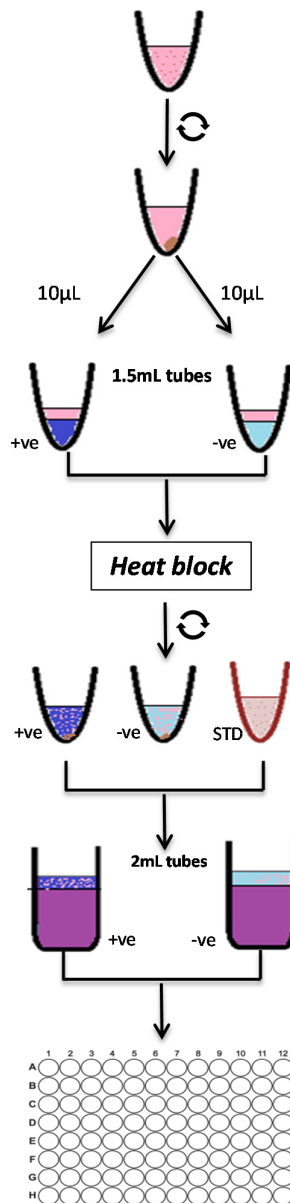
Appendix 1: Glycogen assay optimisation

Enzymatic glycogen content measurement – original protocol

A glycogen assay protocol was obtained from collaborators at the University of Melbourne. This protocol had been previously used extensively to assess the glycogen levels of liver and skeletal muscle tissues. Given that glycogen content and structure are tissue specific, the assay protocol required optimisation and validation for use with cardiac tissues.

In brief, this assay is based on a measurement of the release of glucose from the glycogen molecules within the tissue (Figure. 8.1). The homogenate is incubated with amyloglucosidase (acid- α -glucosidase) to digest the glycogen to release glucose. The sample is then centrifuged to remove unwanted cellular debris. The amount of glucose is measured using a glucose-oxidase reaction solution, which produces a coloured product in proportion to the presence of glucose. The samples are run in triplicate and the intensity of colour is then analysed by absorption of 450nm light. Tissue homogenate is assayed with and without amyloglucosidase (“enzyme-positive” and “enzyme-negative” samples) to obtain a measure of glucose without enzyme-glycogen digestion (i.e. free tissue glucose), and a measure of total glucose after glycogen digestion (i.e. free tissue glucose + glucose released from glycogen). The difference between these two values represents the glucose released from glycogen digestion and is therefore a measure of the total glycogen content of the heart. Glycogen levels are presented in nM glycosyl units and normalised to respective protein content, determined by the Lowry method.

Original Glycogen Assay Protocol



A. Pipette 50µL of heart homogenate

B. 8000g/RCF for 2mins to remove cell debris (*Spin A*)

C. Take two aliquots of 10µL of supernatant into separate tubes

D. Add 100µL master mix consisting of NaAc (pH 6.0), H₂O, +/- amyloglucosidase (converts glycogen to glucose)

E. Mix well and incubate at 50°C for 30minutes

F. Prepare standards (0, 20, 50, 100, 200µM glucose)

G. 16,000g RCF for 2 minutes at 4°C.

H. Pipette 100µL of supernatant /standards into a 2mL tube containing 900µL of PGO enzyme preparation (glucose oxidase/peroxidase + o-dianisidine).

I. Incubate PGO and supernatant solution in the dark for 30minutes at room temperature to complete following reactions:
 Glucose → gluconic acid + H₂O₂
 o-dianisidine is oxidised by H₂O₂ and turns brown in colour

J. Pipette 250µL triplicates of PGO & sample solution according to plate design into 96 well ELISA plate.

K. Measure spectrometric absorbance at 450nm

Opt #3 : Addition of triton
Opt #4 : Addition of syringe pass

Opt #5 : Sample recovery and requirement of initial spin

Opt #2 : Increasing sample input vol.

Opt #6 : Increasing enzyme incubation period

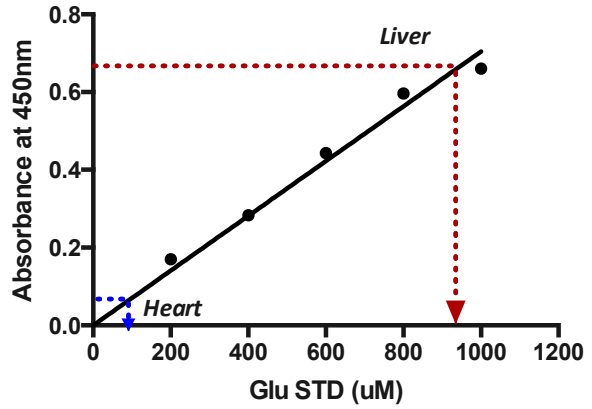
Opt #1 : Detecting glycogen readout range in heart homog

Opt #7 : Assay reproducibility

Figure 8.1: Original glycogen assay protocol.

Glycogen assay optimisations key questions

1. Is glycogen detectable through enzymatic measurement in heart homogenates?
2. Does increasing initial sample input increase glucose absorbance in the heart?
3. Does addition of the detergent triton increase glucose absorbance in the heart?
4. Does addition of a syringe pass step yield higher cardiac glucose absorbance?
5. Does an initial spin A of crude homogenate increase cardiac glucose absorbance?
6. Does increasing the enzyme incubation period increase cardiac glycogen absorbance?
7. Is the optimised glycogen assay protocol for cardiac homogenates reproducible?

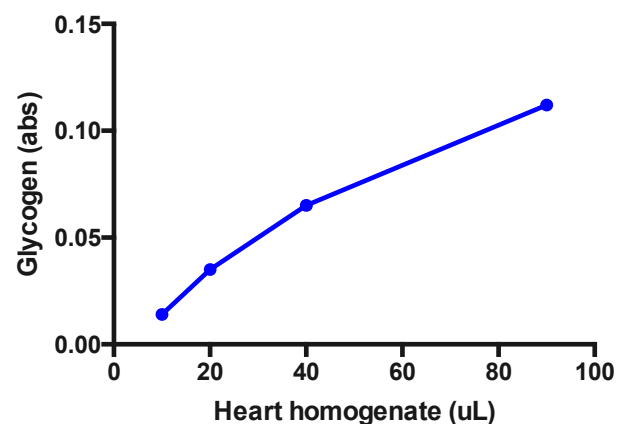
Question (1) :	Is glycogen detectable through enzymatic measurement in heart homogenates?
Hypothesis :	Glycogen present in the myocardium should be detectable through the addition of amyloglucosidase, which breaks down glycogen residues in the heart to glucose. Subtracting free glucose (sample processed without enzyme) from total glucose (sample processed with enzyme) will yield absolute glycogen levels. Glycogen content in the heart is likely to be lower than in the liver, and assay conditions may require optimising to achieve optimal detection.
Experiment :	To enzymatically measure glycogen content in mice heart homogenate with liver as positive control
Results :	 Absorbance values for total glucose in liver (positive control) is 0.65 and in heart homogenate is 0.075
Conclusion :	Yes, glycogen levels are measurable using enzymatic assay but the absolute glycogen absorbance (total-free glucose) in the heart is ~10-fold lower compared to absorbance in liver homogenate. Assay needs to be optimised to have readings from heart homogenate fall at a reliable portion of the standard curve. More points between 0 -20 μ M glu to be added in STD curve (<i>Refers to Step F, Figure 8.1</i>).

Question (2) : Does increasing initial sample input increase glucose absorbance in the heart? (*Refers to Step C, Figure 8.1*)

Hypothesis : Increasing sample input of the 10% heart homogenate may increase the difference between total and free glucose values, translating to a higher absolute glycogen readout from the standard curve

Experiment : To investigate whether increasing initial sample input to 20µl, 40µl and 90µl increases glycogen absorbance level?

Results :



Heart homog (µl)	Total glu (abs)	Free glu (abs)	Glycogen (abs)
10	0.080	0.066	0.014
20	0.102	0.067	0.035
40	0.140	0.075	0.065
90	0.212	0.100	0.112

The increases in absorbance values were directly proportional to volume of sample input.

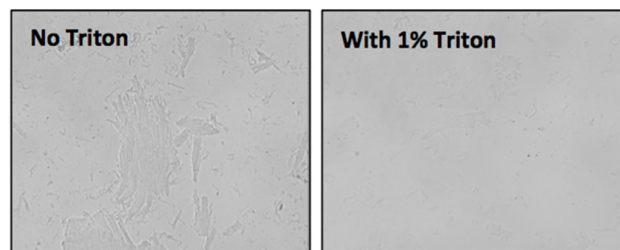
Conclusion : Yes, increasing sample volume increased glycogen absorbance confirming the sensitivity of glycogen detection in the heart. Taking into consideration the volume of 5% homogenate available from mice hearts, a 40µl sample volume was chosen for subsequent assays to obtain absorbance from a more reliable region of the standard curve.

Question (3) : Does addition of the detergent triton increase glucose absorbance in the heart? (*Refers to homogenate preparation before Step A, Figure 8.1*)

Hypothesis : Some glycogen depots in myocardial tissues are enclosed within double membrane vesicles such as phagolysosomes. The addition of 1% Triton may disrupt the double membrane vesicles and release more glycogen during mechanical disruption of heart tissue during homogenisation.

Experiment : To investigate whether addition of 1% Triton to homogenisation buffer improve cellular disruption and increases glycogen absorbance levels

Results :



Addition of triton reduced cell clumping (5µl homogenate viewed at 20x magnification using a light microscope)

Glycogen absorbance increased by 4.3-fold and 4.6-fold with addition of 1% triton in two biological replicates (fold difference calculated against respective tissues homogenised without triton).

Sample #	Without Triton (abs)			With 1% Triton (abs)			Fold diff
	Total	Free	Glycogen	Total	Free	Glycogen	
1	0.094	0.083	0.011	0.114	0.097	0.047	4.3
2	0.084	0.075	0.009	0.117	0.075	0.042	4.6

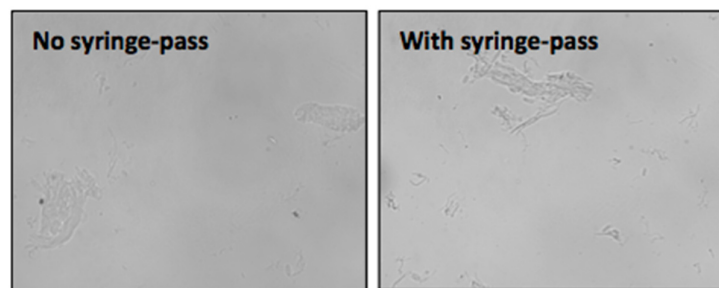
Conclusion : Yes, addition of 1% triton increases glycogen absorbance by ~4.5-fold compared to tissues homogenised without triton.

Question (4) : Does addition of a syringe pass subsequent homogenisation step yield higher cardiac glucose absorbance? (*Refers to homogenate preparation before Step A, Figure 8.1*)

Hypothesis : Passing the homogenate through a syringe to induce further mechanical disruption will avoid cell clumping and provide better access for amyloglucosidase to glycogen

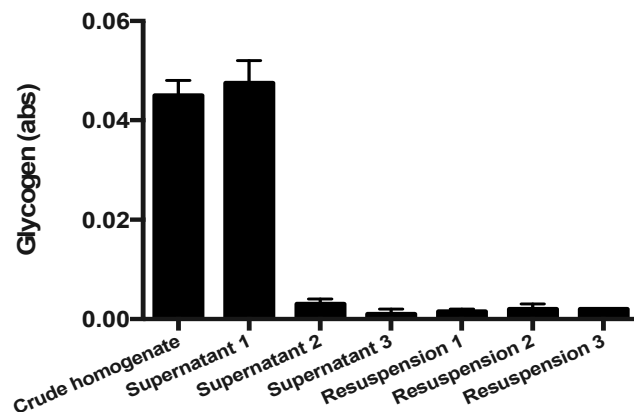
Experiment : To examine whether syringe passing crude homogenate yields a more homogenous mixture.

Results :



No differences in the extent of cell clumping observed visually (20x magnification using light microscope) with syringe-pass introduction.

Conclusion : The time taken to complete this step requires thawed samples to sit for a long time and therefore may affect the integrity of the samples. Therefore, the syringe pass step was not included in the revised protocol.

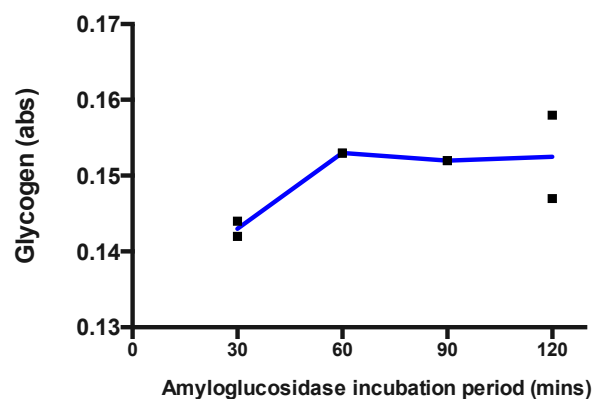
Question (5) :	Does an initial spin of crude homogenate (<i>Refers to Step B, Original glycogen assay protocol, Figure 8.1</i>) increase cardiac glucose absorbance or is glycogen lost in pellet?																	
Hypothesis :	A brief centrifugation of homogenate will pellet crude particles in the homogenate to provide a supernatant which will give better access for enzyme amyloglucosidase to breakdown more glycogen, provided glycogen is not ‘lost’ in the pellet.																	
Experiment :	To compare which sample prep (total homogenate, supernatants and resuspended pellets) will yield the highest glycogen content. Crude homogenate were briefly spun and supernatant 1 was aliquoted while the pellet was resuspended in 1% Tx-homog buffer (resuspension 1). Process was repeated twice to collect supernatant 2, resuspension 2, supernatant 3 and resuspension 3.																	
Results :	 <table><thead><tr><th>Sample Preparation</th><th>Glycogen (abs)</th></tr></thead><tbody><tr><td>Crude homogenate</td><td>0.045</td></tr><tr><td>Supernatant 1</td><td>0.0475</td></tr><tr><td>Supernatant 2</td><td>0.0025</td></tr><tr><td>Supernatant 3</td><td>0.0025</td></tr><tr><td>Resuspension 1</td><td>0.0025</td></tr><tr><td>Resuspension 2</td><td>0.0025</td></tr><tr><td>Resuspension 3</td><td>0.0025</td></tr></tbody></table>	Sample Preparation	Glycogen (abs)	Crude homogenate	0.045	Supernatant 1	0.0475	Supernatant 2	0.0025	Supernatant 3	0.0025	Resuspension 1	0.0025	Resuspension 2	0.0025	Resuspension 3	0.0025	91% of glycogen was recovered within the first two fractions, i.e. crude homogenate and spun supernatant 1. Therefore, only a negligible amount of glycogen was lost in the pellet. The difference in absolute glycogen absorbance values between crude homogenate and supernatant 1 is $0.0475 - 0.045 = 0.0025$.
Sample Preparation	Glycogen (abs)																	
Crude homogenate	0.045																	
Supernatant 1	0.0475																	
Supernatant 2	0.0025																	
Supernatant 3	0.0025																	
Resuspension 1	0.0025																	
Resuspension 2	0.0025																	
Resuspension 3	0.0025																	
Conclusion :	Considering the increase in absorbance with introduction of brief centrifugation of crude homogenate is resulting is minimal, the spin step (Step B) can be removed from the protocol to minimise sample handling and reduce the risk of glycogen degradation.																	

Question (6) : Does increasing the amyloglucosidase incubation period increase glycogen absorbance? (*Refers to Step E, Figure 8.1*)

Hypothesis : A longer incubation time will allow more time for the amyloglucosidase to breakdown glycogen to glucose and increase glycogen detection.

Experiment : To compare glycogen detection with 30, 60, 90 and 120 mins amyloglucosidase incubation time.

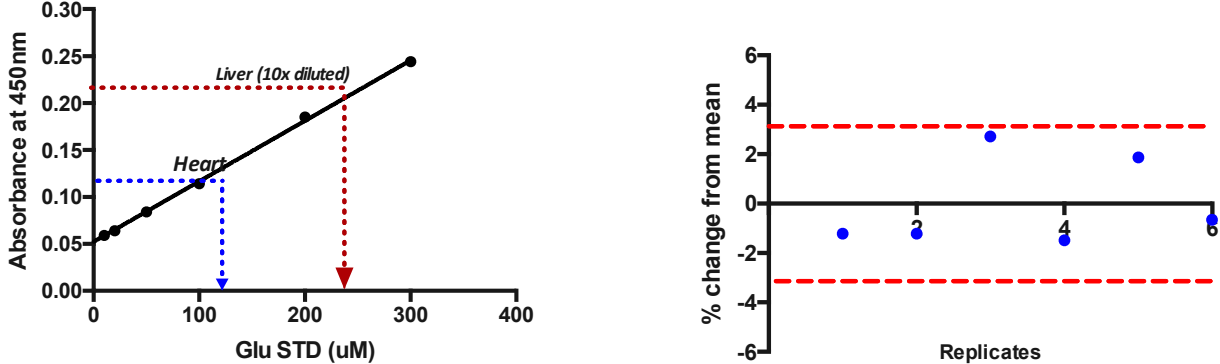
Results :



Increasing enzyme amyloglucosidase incubation time from 30 to 60 mins increased absorbance by 6.4%

No further increases in glycogen absorbance with incubations beyond 60mins, i.e. 90 and 120 mins.

Conclusion : Enzyme amyloglucosidase incubation period of 60 mins is optimal.

Question (7) :	Is the optimised glycogen assay protocol reliable and reproducible?
Hypothesis :	The optimisation changes introduced to the original glycogen assay protocol will produce reliable and reproducible glycogen measurement in heart homogenates (refer key optimisation decisions in the subsequent section for details on changes introduced to original protocol).
Experiment :	To test the reliability and reproducibility of the new optimised glycogen protocol
Results :	 The left graph is a standard curve showing Absorbance at 450 nm on the y-axis (0.00 to 0.30) and Glu STD (uM) on the x-axis (0 to 400). A solid black line represents the standard curve. Two points are marked: 'Heart' at approximately (100, 0.11) and 'Liver (10x diluted)' at approximately (250, 0.22). Dashed blue and red lines connect these points to the x-axis. The right graph shows the percentage change from the mean for 6 replicates on the y-axis (-6 to 6) and Replicates on the x-axis (1 to 6). All data points are blue dots clustered between -1 and 3, with dashed red lines at approximately ±3.2. Glycogen reading for cardiac homogenates are now being taken from a reliable portion of the standard curve. Replicate test using a calibrator heart homogenate yielded less than 3% variability from mean glycogen absorbance between 6 independent measurements.
Conclusion :	The optimised glycogen assay protocol produces both reliable and reproducible glycogen enzymatic measurement in the heart.

Key optimisation conclusions and optimised protocol

1. **Step F:** Standard curve for glycogen measurement in the heart needs to include more points at the lower end of the curve to be able to obtain cardiac glycogen readings from a reliable part of the standard curve.
2. **Step C:** Increasing 5% heart homogenate (mg/vol.) for assaying from 10 μ l to 40 μ l increases glycogen absorbance 4.6-fold.
3. **Homogenate preparation (Before Step A):** Addition of 1% triton to the homogenisation buffer increases glycogen absorbance in heart homogenates ~4.4-fold.
4. **Homogenate preparation (Before Step A):** Syringe pass step was removed from the sample preparation protocol as the extent of cell clumping observed was not markedly different from samples which were not processed using the syringe pass method.
5. **Step B:** An initial spin of crude homogenate step was removed from the protocol as the differences in glycogen absorbance values between crude and spun homogenates were negligible.
6. **Step E:** Enzyme amyloglucosidase incubation period increased to 60 mins from 30 mins as glycogen absorbance is increased by 6.4%.

Enzymatic glycogen assay – optimised protocol for cardiac homogenates

Glucose standards were made from 1mg/ml glucose standard solution (Cat#G6918, pH 3.3, Sigma-Aldrich, MO, USA) and ultrapure H₂O to yield 100µl of 0, 10, 20, 50, 100, 200 and 300 µmol/L stocks.

Amyloglucosidase enzyme-positive and -negative reaction solutions were prepared. The enzyme-positive solutions contained total homogenate, 10⁻⁶M pH6 NaAc, 4.54% amyloglucosidase (#10102857001, Roche, Switzerland), 1% Triton X (Cat#T8787, Sigma-Aldrich, MO, USA) and H₂O to make up to a final volume of 110µl. For non-perfused hearts 1% Triton-X was added to the glycogen assay aliquot at the time of homogenisation so in these samples H₂O was replaced with homogenisation buffer. Optimisation experiments showed that this did not have an effect on the glycogen assay. The enzyme-negative solutions substituted amyloglucosidase with H₂O.

The samples were incubated at 50°C for 60 minutes on a heating block. Incubated samples were then centrifuged at 16,000g for 2 minutes at 4°C. 100µl of supernatant or standard solution was added to 900µl of glucose reaction solution (1capsule of PGO enzyme #P7119, Sigma-Aldrich, MO, USA, 7.9µM o-dianisidine dihydrochloride cat# D3252, Sigma-Aldrich, MO, USA). All samples and standards were mixed well by vortex, covered and incubated at room temperature for 30 minutes. After this incubation samples and standards were pipetted in triplicate into a 96 well plate. A Biorad plate reader (Biorad, CA, USA) was used to read the absorbance at 450nm. A standard curve was generated using the absorbance values from glucose standard solutions and used to determine the concentration of glucose in each sample. Glycogen levels were calculated by the difference between total glucose (enzyme-positive samples) and free tissue glucose (enzyme-negative samples) and normalised against respective protein levels. A schematic of the final glycogen assay protocol has been included on the next page.

Optimised Glycogen Assay Protocol

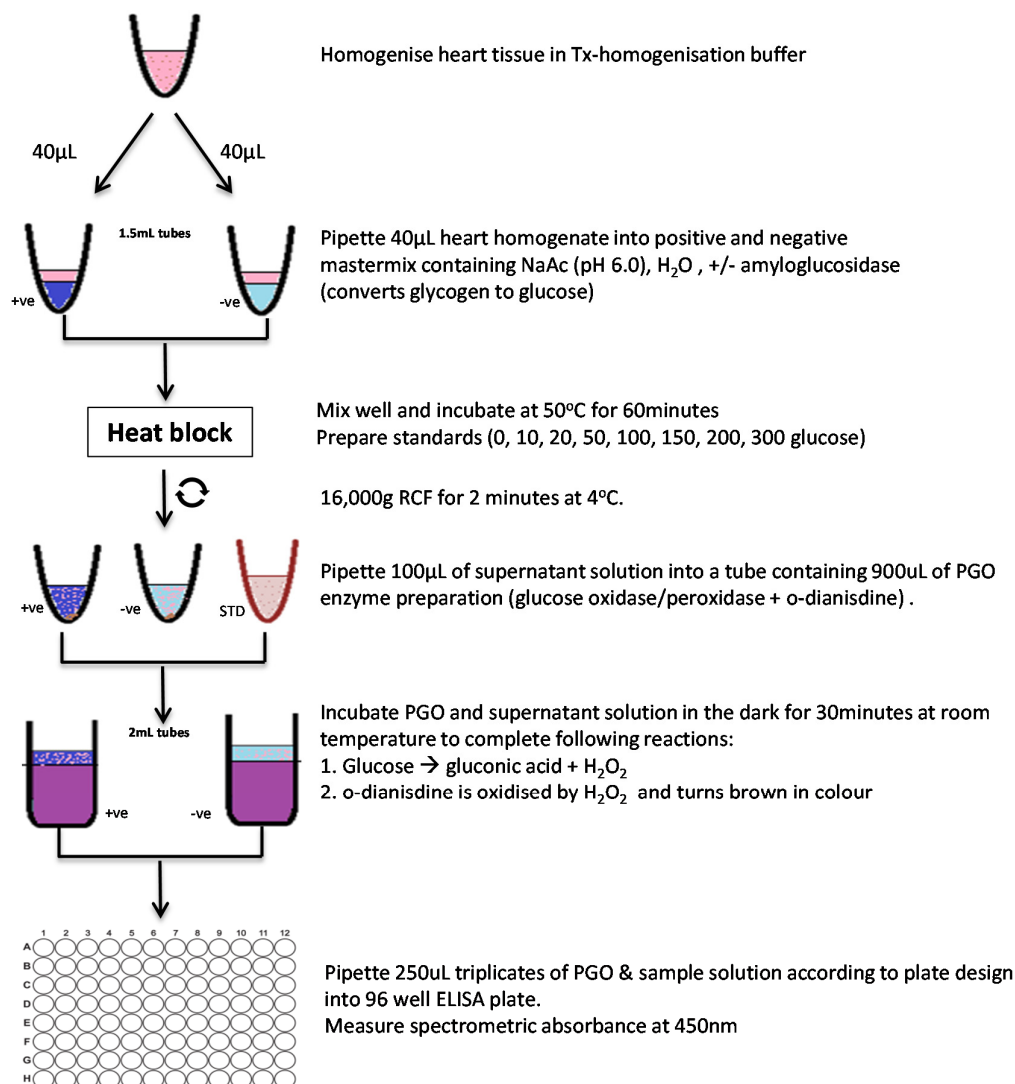


Figure 8.2: Optimised final glycogen assay protocol for cardiac homogenates.

Appendix 2: RNA quantitation and quality control determination

For quality control analysis prior to gene expression profiling using real time RT-PCR, RT² RNA QC PCR Array (Qiagen, CA, USA) was performed on all of the mRNA samples. This was to detect the presence of any reverse-transcription inhibition, PCR amplification inhibition, genomic DNA contamination, false positive signals and to eliminate substandard samples prior to analysis with RT2 Profiler PCR Arrays. A description of the specific controls utilised in this QC plate is given below:

Housekeeping controls - Two housekeeping genes, β -actin (*ACTB*) and *Hypoxanthine Phosphoribosyltransferase 1* (*HPRT1*) were included to predict the expected C_t ranges in subsequent reactions and as an estimation of RNA integrity.

Reverse-transcription control (RTC) - During the reverse-transcription step, a built-in external RNA control is included, which will be detectable in the PCR array. The presence of inhibitors of the reverse-transcription reaction in the RNA samples may also affect the RT of mRNA of interest. A value of less than 5 when $\Delta C_t = C_t^{RTC} - C_t^{PPC+H_2O}$ is calculated is indicative of no contamination.

Positive PCR control (PPC) - The positive PCR control contains a plasmid template with an artificial sequence and primers to detect it in the PCR array. Two controls are characterised with or without cDNA template to test for the presence of PCR inhibitors in the RNA samples. A value of less than 3 when $\Delta C_t = C_t^{PPC+cDNA} - C_t^{PPC+H_2O}$ is calculated denotes no apparent inhibition.

DNA contamination controls - Two methods are used to sensitively detect genomic DNA contamination. The no reverse-transcription control (NRT) mixes primers for a housekeeping gene with total RNA instead of template to test for genomic DNA. Another assay specifically amplifies nontranscribed genomic DNA (gDNA) contamination. Values more than C_t 35 in NRT and gDNA indicate no genomic DNA contamination is present.

No template control (NTC) - A control lacking cDNA template serves as a no template control to test for the introduction of any DNA contamination during experimental setup. An NTC value of more than 35 indicates that there is no evidence of general DNA contamination.

Table 8.1: Analysis of RT² RNA QC PCR Array Results

Sample	Chronic STZ male mouse study						Acute STZ male mouse study						db/db mouse study						Cut-off
ACTB	24.17	23.99	23.56	24.78	23.89	23.64	22.77	22.98	22.73	22.61	22.91	23.04	23.38	24.68	24.14	27.56	24.93	24.62	-
HPRT1	23.42	23.55	23.27	23.44	22.40	21.83	22.36	22.62	22.40	20.89	21.13	21.38	21.26	24.91	23.45	21.76	23.33	22.86	-
RTC	26.64	26.51	26.48	26.50	26.59	26.72	26.79	26.64	26.52	26.63	26.60	26.65	26.36	26.41	26.38	26.43	26.48	26.57	-
PPC	21.17	21.11	21.13	21.13	21.13	21.63	21.51	21.18	21.15	21.32	21.17	21.32	21.22	21.36	21.30	21.33	21.32	21.66	-
GDC	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	35
NRT	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	35
PPC+H2O	21.99	21.72	21.78	21.69	21.66	21.79	21.92	22.25	21.85	22.03	21.83	22.04	22.03	22.15	21.89	22.03	21.78	21.79	-
NTC	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	> 37	35
RT control $\Delta Ct = Ct^{RTC} - Ct^{PPC+H2O}$	4.65	4.79	4.71	4.82	4.93	4.93	4.87	4.39	4.67	4.60	4.77	4.62	4.33	4.26	4.49	4.41	4.70	4.79	5
PPC control $\Delta Ct = Ct^{PPC} - Ct^{PPC+H2O}$	-0.82	-0.61	-0.64	-0.56	-0.53	-0.16	-0.41	-1.07	-0.70	-0.72	-0.65	-0.72	-0.82	-0.79	-0.59	-0.70	-0.46	-0.13	3

Samples included in the QC Array plate were from STZ (8weeks), ACUTE STZ (cardiac) and *db/db* study (3 controls and 3 diabetic samples each). Detectable threshold values in both housekeeping genes (*ACTB* and *HPRT1*) in all RNA samples provide confidence that the RNA is intact. Values of less than Ct 5 and Ct 3 in RT control and PPC control respectively indicate no presence of RT or PCR inhibitors. Given that the Ct values for GDC, NRT and NTC were more than Ct 37, no genomic DNA or general DNA contamination was introduced during the PCR reaction. Therefore, all RNA samples were confirmed to be of good quality for the RT profiling gene expression analysis.

RNA Quantitation results through Nanodrop analysis

RNA purity and concentrations were analysed using spectrometry (Nanodrop, Thermo Fisher Scientific, MA, USA). A 260/280nm ratio of 1.90-2.10 was accepted as “pure” RNA with least contamination of proteins and phenols as the latter absorb strongly at 280nm. Yet, the actual ratios depend on composition of nucleic acids and sample acidity, therefore acceptable range 260/280nm ratio absorbance was extended to 1.9-2.20 (Wilfinger, 1997). In addition, the 260/230nm ratio was also used to determine RNA quality, where values between 2.0-2.2 are as “rule of thumb” considered pure RNA. Remnants of carbohydrates, EDTA and phenols, which absorb at 230nm would result in a ratio of <2.0 indicating contamination in RNA samples. As a marginally higher ratio is indicative of lower contaminants in the RNA samples, the acceptable range for the 260/230nm ratio were extended to 2.0-2.3 (Leninger, 1975). RNA samples that fell marginally outside these acceptable ranges were included in the plate after being confirmed as acceptable quality using the RT² RNA QC PCR Array.

Table 8.2: Nanodrop RNA concentration and purity results

Study	Sample ID	Group	[RNA] ug/ul	260/280 ratio	260/230 ratio
STZ chronic	23.42	Ctrl	1.16	2.14	2.22
	96	Ctrl	1.29	2.18	1.71
	30.155	Ctrl	1.52	2.15	2.25
	23.4	STZ	1.25	2.15	2.19
	31.161	STZ	0.90	2.11	2.27
	29.148	STZ	1.41	2.07	2.35
Fructose	C1	Ctrl	1.06	2.17	2.20
	C1.1	Ctrl	1.08	2.18	1.87
	C3	Ctrl	1.05	2.14	2.21
	F5	Fructose-fed	0.84	2.14	2.23
	F6	Fructose-fed	1.00	2.15	2.19
	F6.1	Fructose-fed	1.11	2.17	2.25
STZ acute (cardiac)	5	Ctrl	1.04	2.13	1.75
	6	Ctrl	1.04	2.13	2.14
	8	Ctrl	1.10	2.14	2.36
	10	Acute STZ	1.09	2.12	2.12
	11	Acute STZ	1.14	2.17	2.25
	12	Acute STZ	1.18	2.14	2.20
STZ acute (skeletal)	5	Ctrl	1.62	2.11	2.22
	6	Ctrl	1.12	2.10	2.02
	8	Ctrl	1.53	2.11	2.19
	10	Acute STZ	1.39	2.10	2.23
	11	Acute STZ	1.14	2.09	2.23
	12	Acute STZ	1.42	2.10	2.26

Note: *db/db* mRNA samples were a gift from Rebecca Ritchie's laboratory at the Baker IDI Diabetic Institute. The *db/db* samples were confirmed to be high quality in the RT QC plate analysis and were thus included in the RT profiling plate.

References

Leninger AL. *Biochemistry*. New York: Worth Publishers, 1975.

Wilfinger WWM, K.; Chomczynsk, P. Effect of ph and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques* 1997 **22**: 474-481.

Appendix 3: Published papers and submitted abstracts

Publications

1. Chandramouli C, Varma U, Stevens E, Stapleton D, Mellor KM, Delbridge LMD. Myocardial glycogen dynamics: new perspectives on disease mechanisms. *Clinical and Experimental Pharmacology and Physiology*. 2015 Apr;42(4):415-25.
2. Reichelt ME, Mellor KM, Bell JR, Chandramouli C, Headrick JP, Delbridge LMD. Sex, sex steroids and diabetic cardiomyopathy: making the case for experimental focus. *Am J Physiol Heart Circ Physiol*. 2013 Sep 15;305(6):H779-92.

Abstracts

3. Chandramouli C, Reichelt ME, Mellor KM, Ritchie RH, Curl CL, Delbridge LMD. Diabetic myocardial pathology is more severe in female than male mice. Proceedings from National Heart Association of Malaysia Meeting, Apr 2016, Poster presentation
4. Chandramouli C, Reichelt ME, Mellor KM, Ritchie RH, Curl CL, Delbridge LMD. Sex-differences in AngII-mediated exacerbation of diabetic myocardial function. Proceedings from the International Society of Heart Research (Australasian Section) Meeting, Melbourne, Aug 2015.
5. Chandramouli C, Reichelt ME, Mellor KM, Ritchie RH, Curl CL, Delbridge LMD. Elevated cardiac AngII influences diabetic myocardial pathology differently in males and females. Proceedings from the Australian Health and Medical Research Congress, Nov 2014. Poster presentation.
6. Reichelt ME, Chandramouli C, Chan, Z, Mellor KM, Ritchie RH, Curl CL, Delbridge LMD. Elevated cardiac AngII leads to structural and mechanical dysfunction in diabetic normotensive mice. Proceedings from the Australian Physiological Society Meeting, Geelong, Dec 2013, Poster presentation.