DOCTOR OF PHILOSOPHY

Targeting macrophages GLP-1 receptor pathways as emerging therapies to ameliorate cardiac remodelling associated with diabetes

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Targeting macrophages GLP-1 receptor pathways as emerging therapies to ameliorate cardiac remodelling associated with diabetes

Rawan Abudalo, MSc. Pharm.

A thesis submitted to the School of Medicine, Dentistry and Biomedical Sciences, Queen’s University Belfast

For the degree of Doctor of Philosophy

November 2019
Declaration

I declare that:

(i) The thesis is not one for which a degree has been or will be conferred by any other university or institution.

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(iii) The work for the thesis is my own work and the materials are submitted by me for another degree or work undertaken by me as part of a research group has been incorporated into the thesis, the extent of the work thus incorporated has been clearly indicated.

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Rawan Abudalo
2019
Abstract

The pathophysiological link between diabetes and cardiovascular disease is complex with compelling evidence indicating that hyperglycaemia, as a hallmark of diabetes, induces cardiac abnormalities specifically associated with inflammation, extracellular matrix (ECM) changes, and diastolic dysfunction, which accelerate heart failure development and progression. Despite active control of blood glucose and cardiovascular risk factors, macrovascular complications remain the main cause of death in type 2 diabetes. Whilst several molecular and cellular mechanisms are known to contribute to cardiac changes in diabetes, inflammation is becoming increasingly recognised as a major regulator of cardiovascular pathophysiology, which is especially prominent in diabetes. Hence, targeting inflammatory pathways may have direct benefit in treatment of cardiovascular disease in diabetes.

Glucagon-like peptide (GLP-1) is an incretin hormone, which confers proven glycaemic control in diabetes, and also demonstrates cardioprotective effects. Indeed, we have recently reported that the GLP-1 analogue, exendin-4, promotes specific benefits on cardiac inflammation and ECM remodelling in a mouse model of experimental diabetes. Besides its direct action to maintain cardiovascular function and indirect action to control cardiovascular risk factors, it is becoming increasingly evident that GLP-1 confers cardioprotective effects through modulation of inflammatory signalling. Therefore, the aim of this thesis was to study the effects of the GLP-1 analogue, liraglutide, in diabetes with a specific focus on cardiac inflammation.

Based on our previous findings, we initially designed modified GLP-1 peptides to specifically target macrophages. As macrophages express the mannose receptor, we hypothesised that addition of mannose to GLP-1 peptides would increase macrophage selectivity, promoting high affinity binding to the cell surface. However, addition of mannose was found to reduce exendin-4-GLP-1R binding and activation. Therefore, we employed poly (lactic-co-glycolic acid) (PLGA) nanoparticles for preferential targeting of liraglutide and exendin-4 to inflammatory cells. Whilst liraglutide-PLGA nanoparticles displayed equivalent GLP-1R binding and activation to native liraglutide in U2OS cells and CHL cells, respectively, they showed lower efficacy with respect to macrophage GLP-1R binding in vitro and in vivo. This observation may be explained by rapid phagocytosis of nanoparticles before the adsorbed liraglutide had opportunity to bind to the GLP-1R. Therefore, an alternate approach involving, for example, selection of mannosylated or polyethylene glycol...
(PEG) PLGA-nanoparticles, may minimise rapid macrophage phagocytosis thereby promoting liraglutide-GLP-1R binding.

The heart consists of different cell types, including cardiomyocytes, fibroblasts, endothelial cells, and infiltrating inflammatory cells, with intercellular communication playing a key role in cardiac remodelling. Cardiac fibroblasts play a particularly important role in diabetes by promoting ECM remodelling and fibrosis, whilst coronary microvascular endothelial cells secrete inflammatory factors that negatively influence cardiac structure and function to induce adverse remodelling. Accumulating evidence suggests that both cardiac fibrosis and endothelial dysfunction in diabetes are driven by infiltrating inflammatory cells, all of which have been shown to be altered by GLP-1 agonists, although cardiac fibroblasts do not express the GLP-1R, highlighting indirect actions which appear to be largely mediated by macrophages. As such, macrophages were treated with high glucose which induced cytokine/chemokine expression, dependent upon NF-κB as a prototypical proinflammatory mediator associated with macrophages infiltration and adhesion, effects which were reversed by liraglutide. Furthermore, liraglutide attenuated fibroblast differentiation and endothelial cell dysfunction induced by incubation with conditioned media from high glucose-treated macrophages, highlighting modulation of paracrine signalling as a likely mechanism underlying its apparent cardioprotective actions.

In order to investigate potential translation of these experimental findings, initial clinical analyses were undertaken in type 2 diabetic patients receiving conventional glycaemic medication and those receiving additional liraglutide. Interestingly, although preliminary in nature, this study highlighted potential modulation of circulating inflammatory cells showing some correlation with cardiac function, which could be subject to modulation by liraglutide.

Taken together, the findings of this thesis have established that macrophage-specific targeting by modified GLP-1 is more complex than anticipated. However, given our in vitro findings indicating that liraglutide modulates macrophage inflammatory autocrine and paracrine signalling, selective macrophage modulation holds clear potential to maximise cardioprotective effects of GLP-1 on ECM remodelling and endothelial dysfunction. In order to establish translational relevance of such experimental data, it is critical to generate comprehensive clinical data in order to support the specific use of GLP-1 based therapies to prevent and reduce adverse cardiac remodelling and dysfunction associated with diabetes.
Publications/Academic Achievements

Manuscripts
Adam J Wilson, Eleanor K Gill, Rawan A Abudalo, Kevin S Edgar, Chris J Watson and David J Grieve: Reactive oxygen species signalling in the diabetic heart: emerging prospect for therapeutic targeting, Heart 2018; 104: 293.


Poster presentations
26th Northern Cardiovascular Research Group Meeting, Newcastle 24th April 2018.
BAS/BSCR Meeting, Manchester 4-5th June 2018.
19th Irish Association of Pharmacologists Annual Meeting 30th November 2018.

Awards
1st place in the poster competition at the Irish Association of Pharmacologists meeting, 30th November 2018
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From September 2016 to November 2019, this thesis was carried out at the Centre of experimental medicine/ School of Medicine, Dentistry and Biomedical Sciences. This PhD degree was an assignment I had wished for a long time, and when I first got Queen’s University offer, I was overwhelmed of happiness. The thesis work has involved a great number of people, whom I am appreciated to and wish to thank them all for their help and support.

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## Table of content

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declaration</td>
<td>ii</td>
</tr>
<tr>
<td>Abstract</td>
<td>iii</td>
</tr>
<tr>
<td>Publications/Academic Achievements</td>
<td>v</td>
</tr>
<tr>
<td>Acknowledgement</td>
<td>vi</td>
</tr>
<tr>
<td>1 General Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Diabetes mellitus</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Definition of diabetes mellitus</td>
<td>1</td>
</tr>
<tr>
<td>1.1.2 Diabetes prevalence</td>
<td>1</td>
</tr>
<tr>
<td>1.1.3 Pathophysiology of diabetes mellitus</td>
<td>1</td>
</tr>
<tr>
<td>1.1.4 Classification of diabetes mellitus</td>
<td>2</td>
</tr>
<tr>
<td>1.1.4.1 Type I diabetes mellitus</td>
<td>2</td>
</tr>
<tr>
<td>1.1.4.2 Type II diabetes mellitus</td>
<td>3</td>
</tr>
<tr>
<td>1.1.4.3 Gestational diabetes</td>
<td>3</td>
</tr>
<tr>
<td>1.1.5 Diabetes complications</td>
<td>4</td>
</tr>
<tr>
<td>1.1.5.1 Macrovascular and microvascular changes in diabetes</td>
<td>4</td>
</tr>
<tr>
<td>1.2 Heart failure and diabetic cardiomyopathy</td>
<td>5</td>
</tr>
<tr>
<td>1.2.1 Correlation between diabetes, cardiovascular disease and heart failure</td>
<td>5</td>
</tr>
<tr>
<td>1.2.2 Heart failure</td>
<td>6</td>
</tr>
<tr>
<td>1.2.2.1 Epidemiology of heart failure</td>
<td>6</td>
</tr>
<tr>
<td>1.2.2.2 Aetiology of heart failure</td>
<td>7</td>
</tr>
<tr>
<td>1.2.2.3 Physiological response to heart failure</td>
<td>7</td>
</tr>
<tr>
<td>1.2.2.4 Stages and management of heart failure</td>
<td>8</td>
</tr>
<tr>
<td>1.2.3 Diabetic cardiomyopathy</td>
<td>10</td>
</tr>
<tr>
<td>1.2.3.1 Definition of diabetic cardiomyopathy</td>
<td>10</td>
</tr>
<tr>
<td>1.2.3.2 Metabolic and structural changes in diabetic cardiomyopathy</td>
<td>11</td>
</tr>
<tr>
<td>1.2.3.2.1 Cardiac structural and functional changes in diabetes mellitus</td>
<td>12</td>
</tr>
<tr>
<td>1.2.3.2.2 Pathological changes in the diabetic heart</td>
<td>13</td>
</tr>
<tr>
<td>1.2.3.3 Characteristic features of the diabetic heart</td>
<td>13</td>
</tr>
<tr>
<td>1.2.3.3.1 Cardiac hypertrophy</td>
<td>14</td>
</tr>
<tr>
<td>1.2.3.3.2 Cardiac necrosis and apoptosis</td>
<td>14</td>
</tr>
<tr>
<td>1.2.3.3.3 Fibrosis</td>
<td>16</td>
</tr>
<tr>
<td>1.2.3.3.4 ECM remodelling</td>
<td>17</td>
</tr>
</tbody>
</table>
1.2.3.4 Pathophysiological triggers of diabetic cardiomyopathy .......... 19
  1.2.3.4.1 Hyperglycaemia ..................................................... 19
  1.2.3.4.2 Impaired myocardial insulin signalling ................................ 21
  1.2.3.4.3 Inappropriate Renin-Angiotensin-Aldosterone System activation ......................................................... 22
  1.2.3.4.4 Cardiac lipotoxicity .......................................................... 23
  1.2.3.4.5 Maladaptive immune modulation ............................................. 24
  1.2.3.4.6 Inappropriate neurohumoral activation ....................................... 25
1.2.3.5 Treatment strategies of diabetic cardiomyopathy .......... 25
  1.2.3.5.1 Lifestyle modification ................................................................. 25
  1.2.3.5.2 Glycaemic control and antidiabetic medications ......................... 26
    1.2.3.5.2.1 Sulfonylureas ................................................................. 26
    1.2.3.5.2.2 Thiazolidinediones ......................................................... 26
    1.2.3.5.2.3 Metformin ................................................................. 27
    1.2.3.5.2.4 Incretin based therapies .................................................. 27
    1.2.3.5.2.5 Sodium-glucose cotransporter 2 inhibitors .................................. 28
  1.2.3.5.3 Conventional therapies for diabetic cardiomyopathy ........ 29
    1.2.3.5.3.1 Lipid-lowering therapies-statins ........................................ 29
    1.2.3.5.3.2 RAAS-inhibition .......................................................... 29
    1.2.3.5.3.3 β-blockers ................................................................. 30
1.3 The incretin axis and glucagon-like peptide-1 (GLP-1) biology .......... 30
  1.3.1 Incretin physiology and effect ...................................................... 31
  1.3.2 GLP-1 metabolism and elimination .................................................. 32
  1.3.3 GLP-1 receptor signalling ......................................................... 32
  1.3.4 Physiological actions of GLP-1 ...................................................... 32
  1.3.5 The incretin system in type 2 diabetes mellitus ................................ 33
  1.3.6 GLP-1 and cardiovascular system .............................................. 34
    1.3.6.1 The effect of GLP-1 on risk factors for cardiovascular disease and diabetes .................................................. 36
    1.3.6.2 GLP-1 and cardiovascular dysfunction ........................................ 38
    1.3.6.3 GLP-1 and cardiac remodelling .............................................. 40
1.4 Thesis aims and objectives .......................................................... 42
2 Materials and methods ................................................................. 44
  2.1 Cell culture ..................................................................................... 44
    2.1.1 Murine macrophages culture ...................................................... 44
      2.1.1.1 RAW 264.7 macrophages ....................................................... 44
      2.1.1.2 Immortalised murine bone marrow macrophages ....................... 45
2.1.1.3 Treatment of mouse macrophages ........................................... 46
2.1.1.4 Harvesting of conditioned media ........................................... 47
2.1.2 Primary mouse cardiac fibroblasts ........................................... 47
2.1.3 Murine 3T3 NIH fibroblasts ........................................... 47
2.1.4 Murine cardiac endothelial cells ........................................... 48
2.1.5 Macrophages conditioned media treatment ................................ 49
2.1.6 Isolation of peripheral blood mononuclear cells (PBMCs) ............. 49
2.2 Reverse-transcription polymerase chain reaction (RT-PCR) ............... 51
2.2.1 Extraction of nucleic acids ........................................... 51
2.2.1.1 Determination of nucleic acid concentration ........................... 51
2.2.1.2 Estimation of RNA quality ........................................... 52
2.2.2 Conversion of RNA to cDNA ........................................... 53
2.2.2.1 DNase Treatment ........................................... 53
2.2.2.2 cDNA synthesis ........................................... 53
2.2.3 Primer design ........................................... 54
2.2.4 Calculation of primers efficiency ........................................... 55
2.2.5 Real-time RT-PCR ........................................... 56
2.2.6 Analysis of Quantitative RT-PCR ........................................... 58
2.3 Western blotting ........................................... 59
2.3.1 Sample preparation ........................................... 59
2.3.2 Protein quantification ........................................... 60
2.3.3 Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) ........................................... 60
2.3.4 Protein transfer ........................................... 61
2.3.5 Immunoblotting with specific antibodies .................................. 62
2.4 Immunocytochemistry ........................................... 63
2.5 Flow cytometry ........................................... 65
2.6 Flexstation (Ca^{2+}) assay ........................................... 66
2.7 Barrier formation assay ........................................... 67
2.8 Statistical analysis ........................................... 67
3 In vitro examination of direct effects of liraglutide on macrophage activation and indirect effects on cardiac fibroblasts and endothelial cells in experimental diabetes ........................................... 68
3.1 Introduction ........................................... 68
3.1.1 Signalling mechanisms underlying inflammation in the diabetic heart68
3.1.2 The cell biology of diabetes-associated cardiac fibrosis .......................... 69
3.1.2.1 Macrophages and immune cells ........................................ 69
3.1.2.2 Cardiac fibroblasts ......................................................... 71
3.1.2.3 Endothelial cells .......................................................... 73
   3.1.2.3.1 Physiology of the endothelium ..................................... 73
   3.1.2.3.2 Diabetes and endothelial dysfunction .............................. 75
3.2 Materials and methods .......................................................... 76
   3.2.1 Drugs used in the study .................................................. 76
      3.2.1.1 Exendin-4 .............................................................. 76
      3.2.1.2 Liraglutide ............................................................. 76
      3.2.1.3 Exendin (9-39) ......................................................... 77
   3.2.2 Expression of GLP-1 receptor (GLP-1R) in mouse macrophages and cardiac fibroblasts ................................................................. 77
   3.2.3 Treatment of macrophages ................................................. 78
   3.2.4 Harvesting of conditioned media ....................................... 78
   3.2.5 Paracrine signalling assessment between RAW cells and cardiac fibroblasts ................................................................................. 79
   3.2.6 Assessment of liraglutide anti-inflammatory action through NF-κB nuclear activation ................................................................. 79
   3.2.7 Identification of molecular anti-inflammatory mechanism of liraglutide action in macrophages .......................................................... 80
   3.2.8 Proteome array ................................................................... 80
   3.2.9 Macrophage migration assay ................................................. 81
   3.2.10 Macrophage adhesion assay ................................................. 81
   3.2.11 Conditioned media experiments .......................................... 82
      3.2.11.1 Evaluation of macrophage paracrine signalling effects on cardiac fibroblasts differentiation and ECM protein expression ...................... 82
      3.2.11.2 Evaluation of macrophage paracrine signalling effects on cardiac fibroblasts calcium handling ......................................................... 82
      3.2.11.3 Assessment of macrophage paracrine signalling effects on endothelial cells activation ................................................................. 83
3.3 Results ................................................................................... 83
   3.3.1 GLP-1R expression was confirmed in mouse macrophages, but not 3T3 fibroblasts ................................................................. 83
   3.3.2 Exendin-4 and liraglutide may modulate RAW cell cytokine and chemokine mRNA expression after exposure to high glucose ...................... 83
   3.3.3 High glucose-treated RAW cells elicit paracrine action on primary cardiac fibroblast differentiation which is modulated by liraglutide but not exendin-4 ................................................................. 86
3.3.4 High glucose-treated immortalised bone marrow-derived macrophages promote paracrine actions on 3T3 fibroblast differentiation ........................................ 86
3.3.5 Liraglutide and exendin-4 treated iBMDM differentially affect paracrine regulation of 3T3 fibroblast differentiation in a dose-dependent manner .......... 91
3.3.6 Liraglutide attenuated hyperglycaemia induced inflammation in iBMDM by inhibiting nuclear NF-κB translocation ........................................ 93
3.3.7 Liraglutide altered the expression of iBMDMs secreted inflammatory cytokines and chemokines in response to high glucose ..................... 93
3.3.8 Liraglutide modulates high glucose induced macrophage migration and adhesion ........................................................................ 96
3.3.9 iBMDM paracrine signalling alters cell differentiation and function and is influenced by high glucose and liraglutide ...................................... 99

3.3.9.1 High glucose-treated iBMDM influence cardiac fibroblast differentiation and ECM protein expression which is altered by liraglutide ... 99
3.3.9.2 Conditioned media from high glucose-treated iBMDM alters cardiac fibroblast calcium signalling and TRPV2 channel expression which is influenced by liraglutide ........................................ 105
3.3.9.3 Conditioned media from high glucose-treated iBMDM alters cardiac endothelial cell function which is influenced by liraglutide .......... 109
3.4 Discussion ............................................................................. 114

4 Development of cell-targeted GLP-1 peptides with specific affinity for macrophages ........................................................................ 122
4.1 Introduction ............................................................................ 122
4.1.1 Targeted drug delivery .......................................................... 122
4.1.2 Receptor–based approach to target macrophages .................... 122
4.1.3 Nanocarrier-based macrophage targeting ................................ 123
4.1.3.1 PLGA nanoparticles for drug delivery ............................... 124
4.1.4 GLP-1 receptor characteristics .............................................. 125
4.1.4.1 G protein-coupled receptors (GPCRs) - structure and activation .................................................................................. 125
4.1.4.2 Glucagon like peptide-1 receptor (GLP-1R) ...................... 127
4.1.4.3 Signal transduction of GLP-1 ........................................... 127
4.1.5 Aims of this chapter ................................................................ 129
4.2 Materials and methods ............................................................ 130
4.2.1 Modification of exendin-4 by addition of mannose .................. 130
4.2.2 Modification of peptides by attachment to PLGA nanoparticles ...... 130
4.2.2.1 Synthesis of PLGA-based nanoparticle emulsion ................. 131
4.2.2.2 Nanoparticle washing ...................................................... 132
4.2.2.3 Addition of liraglutide or exendin-4 ................................. 132
4.2.2.4 Peptide-nanoparticle quantification .................................................. 133
4.2.3 Evaluation of modified GLP-1 peptides receptor binding affinity, and
receptor activation ......................................................................................... 133
4.2.3.1 Redistribution assay ........................................................................... 133
  4.2.3.1.1 U2OS cell culture ........................................................................ 133
  4.2.3.1.2 U2OS cells treatment for redistribution assay .......................... 133
4.2.3.2 cAMP assay ....................................................................................... 134
  4.2.3.2.1 CHL cell culture .......................................................................... 134
  4.2.3.2.2 Determination of cellular cAMP using cAMP assay kit .......... 135
4.2.4 Assessment of liraglutide-modified peptides macrophages specific
affinity by immunocytochemistry ............................................................... 135
4.2.5 Assessment of liraglutide-modified peptides macrophage-specific
affinity by live cell imaging ......................................................................... 136
4.2.6 Evaluation of modified GLP-1 peptides specific affinity for
macrophages by flow cytometry ................................................................. 136
  4.2.6.1 Assessment of mannose-exendin-4 GLP-1R affinity ................. 136
  4.2.6.2 Optimisation of anti GLP-1 antibody concentration for flow
cytometry ................................................................................................. 137
  4.2.6.3 iBMDM cell permeabilisation to detect intracellular GLP-1R .... 137
  4.2.6.4 iBMDM cell permeabilisation and staining with isotype controls to
assess antibody specificity. ....................................................................... 138
  4.2.6.5 Evaluation of GLP-1 APC antibody staining efficacy .............. 138
  4.2.6.6 Assessment of liraglutide nanoparticle affinity for macrophages
...................................................................................................................... 138
  4.2.6.7 Flow cytometry analysis of liraglutide and liraglutide nanoparticles
to assess their affinity to murine-derived peritoneal inflammatory cells..... 139
4.3 Statistical analysis ................................................................................... 141
4.4 Results ..................................................................................................... 141
  4.4.1 Effect of GLP-1 agonists and modified peptides on GLP-1R
translocation ............................................................................................... 141
  4.4.2 Stimulation of cAMP production by GLP-1 agonists and modified
peptides ....................................................................................................... 145
  4.4.3 Immunostaining revealed uptake of PLGA nanoparticles by
macrophages ............................................................................................... 147
  4.4.4 Flow cytometry analysis of in vitro macrophage targeting by liraglutide-
PLGA nanoparticles .................................................................................... 147
  4.4.5 Flow cytometry analysis of in vivo inflammatory cell targeting by
liraglutide-PLGA nanoparticles .................................................................. 153
4.5 Discussion ............................................................................................... 157
5 Investigation of potential effects of liraglutide on cardiac function and circulating inflammatory cell profile in type 2 diabetic patients............... 161

5.1 Introduction.............................................................................................................. 161

5.2 Materials and methods .......................................................................................... 165

5.2.1 Research design..................................................................................................... 165

5.2.2 Assessment of cardiac function by echocardiography........................................ 165

5.2.3 Patient blood processing ........................................................................................ 168

5.2.3.1 Assessment of liraglutide treatment on inflammatory marker expression at baseline and after stimulation with LPS ......................... 168

5.2.3.2 Collection of patient plasma................................................................................. 168

5.2.3.3 Isolation of peripheral blood mononuclear cells (PBMCs) ................... 168

5.2.3.4 Assessment of circulating inflammatory cell profiles in isolated PBMCs by flow cytometry ................................................................. 168

5.2.4 Statistical analysis ................................................................................................. 169

5.3 Results ..................................................................................................................... 169

5.3.1 Clinical data and medications history................................................................. 170

5.3.2 Echocardiography data........................................................................................ 173

5.3.3 Correlation between echocardiographic indices and metabolic profile ............ 179

5.3.4 Inflammatory cell profiles in PBMCs isolated from type 2 diabetic patients .......... 183

5.4 Correlation between circulating inflammatory cells and metabolic profile ......... 186

5.4.1 Correlation between circulating inflammatory cells and cardiac function ........ 189

5.5 Discussion ............................................................................................................... 195

6 General discussion ..................................................................................................... 200

6.1 Study limitations and future work ............................................................................. 206

References ..................................................................................................................... 209
### TABLE OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>ACA/AHA stages and NYHA classification of heart failure</td>
</tr>
<tr>
<td>1-2</td>
<td>Stages of Heart Failure and Treatment Options. Adapted from</td>
</tr>
<tr>
<td>1-3</td>
<td>Potential mechanisms implicated in the development of cardiac dysfunction and diabetic cardiomyopathy.</td>
</tr>
<tr>
<td>1-4</td>
<td>Trans-mitral valve spectral Doppler flow pattern of 3 subjects</td>
</tr>
<tr>
<td>1-5</td>
<td>Overview of cardiomyocyte and non-cardiomyocyte changes during cardiac remodelling</td>
</tr>
<tr>
<td>1-6</td>
<td>The role of MMPs and TIMPs in cardiac remodelling</td>
</tr>
<tr>
<td>1-7</td>
<td>Physiological actions of GLP-1 receptor activation</td>
</tr>
<tr>
<td>1-8</td>
<td>GLP-1 response after glucose load</td>
</tr>
<tr>
<td>1-9</td>
<td>Potentiating GLP-1 action by two main classes of drugs: GLP-1 receptor agonists, DPP-4 inhibitors</td>
</tr>
<tr>
<td>1-10</td>
<td>Direct and indirect cardioprotective mechanisms of GLP-1R agonists</td>
</tr>
<tr>
<td>2-1</td>
<td>Representative image of RAW 264.7 cells</td>
</tr>
<tr>
<td>2-2</td>
<td>Representative image of immortalised murine bone marrow derived macrophages(iBMDM)</td>
</tr>
<tr>
<td>2-3</td>
<td>Representative image of NIH 3T3 fibroblasts</td>
</tr>
<tr>
<td>2-4</td>
<td>Representative images of immortalised cardiac mouse endothelial cells</td>
</tr>
<tr>
<td>2-5</td>
<td>Whole blood layered onto a density gradient medium for PBMCs isolation</td>
</tr>
<tr>
<td>2-6</td>
<td>Representative image of PBMCs after isolation under light microscope, 20X magnification</td>
</tr>
<tr>
<td>2-7</td>
<td>RNA quality analysis using agarose gel electrophoresis</td>
</tr>
<tr>
<td>2-8</td>
<td>Mechanism of SYBR® Green interaction with DNA</td>
</tr>
<tr>
<td>2-9</td>
<td>Representative setup of transfer sandwich cassette for western blotting protein transfer phase</td>
</tr>
<tr>
<td>2-10</td>
<td>Schematic representation of immunocytochemistry protocol</td>
</tr>
<tr>
<td>3-1</td>
<td>Molecular and cellular mechanisms underlying inflammation in diabetic cardiomyopathy and specific involvement of NF-κB and the renin-angiotensin-aldosterone system (RAAS)</td>
</tr>
<tr>
<td>3-2</td>
<td>The role of macrophage inflammatory mediators in cardiac fibrosis</td>
</tr>
<tr>
<td>3-3</td>
<td>Pleiotropic functions of cardiac fibroblasts</td>
</tr>
<tr>
<td>3-4</td>
<td>Fibroblast proliferation and differentiation to myofibroblasts</td>
</tr>
</tbody>
</table>
Figure 3-5: Overview of characteristics of normal endothelium function and dysfunction ................................................................. 73
Figure 3-6 The molecular structure of GLP-1 peptides ........................................ 77
Figure 3-7: Representative images of GLP-1R expression in mouse macrophages and 3T3 fibroblasts. ............................................................. 85
Figure 3-8: Effect of exendin-4, liraglutide on proinflammatory cytokines mRNA expression in RAW cell macrophages incubated in high glucose for 24h, 48h, 72h and 96h. .................................................... 87
Figure 3-9: Effect of exendin-4, liraglutide on anti-inflammatory cytokines and chemokines mRNA expression in RAW cell macrophages incubated in high glucose for 24h, 48h, 72h and 96h. Error! Bookmark not defined.
Figure 3-10: Effect of RAW cell conditioned media on differentiation of primary mouse cardiac fibroblasts. ................................................. 89
Figure 3-11: Effect of high glucose-treated macrophage-conditioned media on 3T3 fibroblast differentiation ......................................................... 90
Figure 3-12: Effect of liraglutide and exendin-4 on 3T3 fibroblast differentiation induced by conditioned media collected from high glucose-treated iBMDM. ........ 92
Figure 3-13: Liraglutide prevents high glucose-induced inflammatory signalling in mouse immortalised bone marrow macrophages through modulation of nuclear NF-κB activation. ......................................................... 94
Figure 3-14: Cytokine/chemokine proteome array blots exposed to 48h iBMDM-conditioned media with quantification of protein expression using HLIImage++ software ................................................................. 95
Figure 3-15: High glucose-induced iBMDM migration is modulated by liraglutide... 97
Figure 3-16: High glucose-induced iBMDM adhesion is modulated by liraglutide... 98
Figure 3-17: Effect of high glucose-treated iBMDM conditioned media on mRNA expression of activated 3T3 fibroblasts and the influence of GLP-1 signalling. .... 101
Figure 3-18: Effect of high glucose macrophage conditioned media on 3T3 fibroblast profibrotic protein expression and the influence of GLP-1 signalling. ................. 102
Figure 3-19: Representative immunocytochemistry images of 3T3 fibroblast activation markers after treatment with high glucose macrophage conditioned media with or without liragulide and exendin (9-39) ............................................................. 103
Figure 3-20: Effect of high glucose macrophage conditioned media on 3T3 fibroblast connexions expression and the influence of GLP-1R signalling. ................. 104
Figure 3-21: Effect of high glucose macrophage-conditioned media and liraglutide on ionomycin and ATP-induced calcium release in 3T3 fibroblasts. ................. 107
Figure 3-22: Effect of high glucose macrophage-conditioned media and liraglutide on TRPV2 channel expression in 3T3 fibroblasts. ......................................................... 108
Figure 3-23: Effect of high glucose and liraglutide iBMDM conditioned media on cardiac endothelial cell mRNA expression. ................................................................. 110
Figure 3-24: Effect of high glucose macrophage-conditioned media on murine cardiac endothelial cells adhesion molecule protein expression and the influence of liraglutide. ................................................................................................................... 111
Figure 3-25: Effect of high glucose macrophage-conditioned media on murine cardiac endothelial cell sirtuin-1 protein expression and the influence of liraglutide. .................................................................................................................................................... 112
Figure 3-26: Effect of high glucose macrophage-conditioned media on cardiac mouse endothelial cells tight junction Zonula occludens-1 (ZO-1) protein expression and the influence of liraglutide. ....................................................................................................................... 113
Figure 3-27: High glucose iBMDM media alters endothelial barrier function which is improved by liraglutide.............................................................. 114
Figure 4-1: Chemical Structure of Poly (Lactic-Coglycolic Acid) (PLGA) polymer. 123
Figure 4-2: Hydrolysis of PLGA polymer to lactic acid and glycolic acid........... 124
Figure 4-3: Activation mechanism of the GLP-1 receptor.................................. 128
Figure 4-4: Proposed mechanisms of GLP-1R trafficking after GLP-1 agonist stimulation in BRIN-BD11 pancreatic cells................................................. 129
Figure 4-5: Exendin-4 peptide after addition of mannose sugar. ....................... 130
Figure 4-6: Liraglutide modification by attachment to PLGA nanoparticles........ 131
Figure 4-7: Exendin-4 modification by PLGA-NHS nanoparticles.................... 132
Figure 4-8: Time dependent stimulation of human GLP-1 receptor and receptor internalisation by GLP-1, compound 2 and compound B......................... 138
Figure 4-9: Effect of GLP-1, exendin-4, and mannose-exendin-4 on GLP-1R internalisation in U20S cells................................................................. 142
Figure 4-10: Effect of exendin-4, and exendin-4-NHS nanoparticles on GLP-1R internalisation in U20S cells.......................................................... 143
Figure 4-11: Effect of liraglutide and liraglutide nanoparticles on GLP-1R internalisation in U20S cells................................................................. 144
Figure 4-12: Induction of cAMP production by GLP-1 peptides and nanoparticles. ............................................................................................................ 146
Figure 4-13: Liraglutide adsorbed PLGA nanoparticles stimulate the GLP-1R and induce cAMP. ............................................................................................................. 146
Figure 4-14: Representative images of GLP-1 peptide binding to iBMDM GLP-1R.. ................................................................. 148
Figure 4-15: Representative live cell images of iBMDM after treatment with liraglutide and nanoparticles.. .................................................. 148
Figure 4-16: Flow cytometry analysis of exendin-4 and mannose-exendin-4 treated macrophages........................................................................ 149
Figure 4-17: Optimisation of FITC anti-GLP-1 antibody concentration for flow cytometry................................................................. 151
Figure 4-18: Detection of liraglutide-receptor complex by flow cytometry after cell permeabilization........................................................................ 151
Figure 4-19: Investigation of anti-GLP-1 antibody specificity by flow cytometry... 152
Figure 4-20: Investigation of anti-GLP-1 antibody specificity in iBMDM by flow cytometry using FITC anti-GLP-1 antibody and its isotype control. ....................... 152
Figure 4-21: Assessment of GLP-1 APC conjugated antibody staining efficacy.. 154
Figure 4-22: Assessment of in vitro iBMDM targeting by liraglutide and lirglutide nanoparticles. ........................................................................... 154
Figure 4-23: Assessment of in vivo macrophages targeting by liraglutide, liraglutide rhodamine-PLGA nanoparticles and blank nanoparticles in 12-week LPS-injected mice.................................................................................. 155
Figure 4-24: Assessment of in vivo neutrophils targeting by liraglutide, liraglutide rhodamine-PLGA nanoparticles and blank nanoparticles in 12-week LPS-injected mice.................................................................................. 155
Figure 4-25: Assessment of in vivo monocytes targeting by liraglutide, liraglutide rhodamine-PLGA nanoparticles and blank nanoparticles in 12-week LPS-injected mice.................................................................................. 156
Figure 4-26: Geometric mean of fluorescence intensity for liraglutide, liraglutide-PLGA nanoparticle and blank nanoparticle uptake by peritoneal inflammatory cells in vivo............................................................................... 156
Figure 4-27: Nanoparticles modification strategy to target macrophages. ........ 160
Figure 5-1: Design of the LEAD trials incorporating the use of liraglutide as a monotherapy or in combination with another antidiabetic medications............ 163
Figure 5-2: Schematic diagram illustrating the clinical study design.................. 167
Figure 5-3: Clinical characteristics between control patients receiving conventional antidiabetic medication and patients receiving additional liraglutide............. 172
Figure 5-4: Echocardiographic measurements to assess left ventricular diastolic function and LV filling pressure................................................................. 174
Figure 5-5: Echocardiographic measurements to assess chambers dimension ... 176
Figure 5-6: Echocardiographic measurements to assess left ventricular systolic function................................................................. 176
Figure 5-7: Echocardiographic measurements which are used to diagnose left ventricular hypertrophy.............................................. 177
Figure 5-8: Correlation of HbA1c to echocardiographic functional indices......... 180
Figure 5-9 Correlation of BMI to echocardiographic functional indices .......... 180
Figure 5-10: Correlation of circulating lipid profiles to echocardiographic functional indices.......................................................... 181
Figure 5-11: Correlation of systolic and diastolic blood pressure to echocardiographic functional indices......................................... 182
Figure 5-12 Flow cytometry analysis to detect different subsets of circulating immune cells in PBMCs .............................................. 184
Figure 5-13 Flow cytometry analysis to detect different subsets of circulating immune cells in PBMCs.............................................. 185
Figure 5-14: Frequency of inflammatory cells in PBMCs isolated from type 2 diabetic patients................................................................. 186
Figure 5-15 Relationship between HbA1c and different inflammatory cells subsets................................................................. 187
Figure 5-16 Relationship between BMI and different inflammatory cells subsets. 188
Figure 5-17 Correlation between macrophages and different echocardiographic indices................................................................. 190
Figure 5-18 Correlation between monocytes and different echocardiographic indices................................................................. 190
Figure 5-19 Correlation between T-lymphocytes and different echocardiographic indices................................................................. 190
Figure 5-20 Correlation between natural killers T-lymphocytes (NKT-cells) and different echocardiographic indices................................. 190
Figure 5-21 Correlation between B-lymphocytes and different echocardiographic indices................................................................. 190
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Late mitral flow velocity</td>
</tr>
<tr>
<td>ACA</td>
<td>American College of Cardiology</td>
</tr>
<tr>
<td>ACCORD</td>
<td>Action to Control Cardiovascular Risk in Diabetes</td>
</tr>
<tr>
<td>ACEIs</td>
<td>Angiotensin-converting enzyme inhibitors</td>
</tr>
<tr>
<td>ADVANCE</td>
<td>Preterax and Diamicron-MR Controlled Evaluation</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end-product</td>
</tr>
<tr>
<td>AHA</td>
<td>American Heart Association</td>
</tr>
<tr>
<td>AMPK</td>
<td>Monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ARBs</td>
<td>Angiotensin-receptor blockers</td>
</tr>
<tr>
<td>AT1</td>
<td>Angiotensin II receptor 1</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BNP</td>
<td>Brain natriuretic peptide</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic-Adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFB</td>
<td>Cardiac fibroblasts</td>
</tr>
<tr>
<td>CHL cells</td>
<td>Chinese hamster lung fibroblasts</td>
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<td>CPA</td>
<td>Cyclopiazonic acid</td>
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<tr>
<td>CREB</td>
<td>cAMP-responsive element binding-protein</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>DBP</td>
<td>Diastolic Blood Pressure</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>DCM</td>
<td>dicholoromethane</td>
</tr>
<tr>
<td>DEPC-H2O</td>
<td>Diethyl pyrocarbonate-H2O</td>
</tr>
<tr>
<td>DG</td>
<td>D-glucose</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate-buffered saline</td>
</tr>
<tr>
<td>DPP-4</td>
<td>Dipeptidyl Peptidase-4</td>
</tr>
<tr>
<td>E</td>
<td>Early mitral flow velocity</td>
</tr>
<tr>
<td>e´</td>
<td>Velocity of the early tissue Doppler signal</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection Fraction</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxidase synthase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
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<tr>
<td>Ex(9-39)</td>
<td>Exendin(9-39)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>------------------------------------</td>
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<td>Ex-4</td>
<td>Exendin-4</td>
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<tr>
<td>FACs</td>
<td>Flow cytometry staining buffer</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FR-β</td>
<td>folate receptor-β</td>
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<tr>
<td>GLP-1</td>
<td>Glucagon like peptide-1</td>
</tr>
<tr>
<td>GLP-1R</td>
<td>GLP-1 receptor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIS</td>
<td>Heat inactivated serum</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>Hydroxy-3-methylglutaryl coenzyme A</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IDDM</td>
<td>Insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interlukin-6</td>
</tr>
<tr>
<td>iBMDM</td>
<td>Immortalised bone marrow-derived macrophages</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate 1</td>
</tr>
<tr>
<td>IVRT</td>
<td>Isovolumetric ventricular relaxation time</td>
</tr>
<tr>
<td>IVSD</td>
<td>Interventricular septal diameter</td>
</tr>
<tr>
<td>L</td>
<td>Liraglutide</td>
</tr>
<tr>
<td>LA</td>
<td>Left atrium</td>
</tr>
<tr>
<td>LAVi</td>
<td>Left atrium volume index</td>
</tr>
<tr>
<td>LEAD</td>
<td>Liraglutide Effect and Action in Diabetes</td>
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<tr>
<td>LEADER</td>
<td>Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results</td>
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<tr>
<td>LG</td>
<td>L-glucose</td>
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<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>LVEDD</td>
<td>Left ventricular end-diastolic dimension</td>
</tr>
<tr>
<td>LVEF</td>
<td>Left ventricular ejection fraction</td>
</tr>
<tr>
<td>LVESD</td>
<td>Left ventricular end-systolic dimension</td>
</tr>
<tr>
<td>LVH</td>
<td>Left ventricular hypertrophy</td>
</tr>
<tr>
<td>LVPWT</td>
<td>Left ventricular posterior wall thickness</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>MCEC</td>
<td>Mouse cardiac endothelial cells</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino) ethanesulfonic acid</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
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<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
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<tr>
<td>NF-κB</td>
<td>kappa-light-chain-enhancer of activated B</td>
</tr>
<tr>
<td>NIDDM</td>
<td>non-insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NYHA</td>
<td>New York Heart Association</td>
</tr>
<tr>
<td>PA-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCI</td>
<td>Percutaneous coronary intervention</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGf2</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly (lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PLGA-NHS</td>
<td>poly (lactic-co-glycolic acid)/n-hydroxysuccinimide.</td>
</tr>
<tr>
<td>PPAR-α</td>
<td>peroxisome proliferator-activated receptor-α</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RAAS</td>
<td>renin-angiotensin-aldosterone system</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor of advanced glycation endproducts</td>
</tr>
<tr>
<td>REACH</td>
<td>Reduction of Atherothrombosis for Continued Health</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio Immuno Precipitation Assay</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic Blood Pressure</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Sec</td>
<td>second</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Siruin-1</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamime</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Tissue inhibitors of metalloproteinases</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>t-PA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
</tr>
<tr>
<td>VADT</td>
<td>Veterans Affairs Diabetes Trial</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscles cells</td>
</tr>
<tr>
<td>Vwf</td>
<td>Von Willebrand factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>αSMA</td>
<td>alpha-smooth muscle actin</td>
</tr>
</tbody>
</table>
**LIST OF TABLES**

Table 2-1: cDNA synthesis protocol ................................................................. 54  
Table 2-2: Factors to be considered for designing primers adapted from: .......... 55  
Table 2-3: Example of serial dilution to get standard curve of 4 samples .......... 55  
Table 2-4: List of primers used in RT-PCR .......................................................... 58  
Table 2-5: RT-PCR protocol used throughout this thesis ................................. 58  
Table 2-6: composition of RIPA buffer .............................................................. 60  
Table 2-7: Composition of resolving and stacking gels for SDS-PAGE ............... 61  
Table 2-8: List of antibodies used for western blotting .................................... 63  
Table 2-9: List of antibodies used for immunocytochemistry ............................ 65  
Table 3-1: Overview characteristics of endothelium function and dysfunction ...... 74  
Table 4-1: Antibodies used for flow cytometry to detect peritoneal cell population 141  
Table 5-1: Antibodies used for inflammatory profiling by flow cytometry .......... 169  
Table 5-2: Demographic and clinical characteristics of all recruited patients ...... 171  
Table 5-3: Calculation of LV fractional shortening .............................................. 175  
Table 5-4: Calculation of IVSD/LVPWT ratio ..................................................... 177  
Table 5-5: Baseline echocardiographic measurements ...................................... 178
1 General Introduction

1.1 Diabetes mellitus

1.1.1 Definition of diabetes mellitus
Diabetes mellitus is a chronic metabolic illness characterized by high blood glucose concentration which results from a defect in the body’s ability to produce and/or utilize insulin (Gutteridge, 1999). This occurs due to either a pathogenic process that involves pancreatic β-cell destruction with consequent insulin deficiency (type 1 diabetes mellitus) or tissue receptor resistance to insulin action (type 2 diabetes mellitus) with inadequate insulin release. As a result, abnormalities in carbohydrate, protein and fat metabolism induce characteristic symptoms, such as polydipsia (excessive thirst), polyuria (frequent urination), weight loss and blurred vision (Gutteridge, 1999).

1.1.2 Diabetes prevalence
During the last 20 years, diabetes prevalence has been increasing dramatically worldwide and is now considered as a major public health problem. In 2017 the number of people with diagnosed diabetes were 451 million which expected to be 693 million in 2045 (Cho et al., 2018). Notably, middle- and low-income countries show a faster increase in prevalence than other countries, largely due to adoption of a western diet and lifestyle (Forouzanfar et al., 2016).

It is particularly worrying that diabetes is predicted to be the seventh main cause of death in 2030 according to WHO (Mathers and Loncar, 2006) with more than 3 million people currently living in the UK with diagnosed diabetes (Diabetes UK, 2017) and an additional ~850,000 people with undiagnosed diabetes (Chumm and Smith, 1975).

1.1.3 Pathophysiology of diabetes mellitus
Understanding the pathophysiology of diabetes relies on detailed knowledge of carbohydrate metabolism and insulin action. When food is consumed, it is digested and broken to small particles to be absorbed into the circulation. Carbohydrates and sugars are broken down to glucose which is either utilized by cells as a source of energy or stored as glycogen.
In a healthy person, insulin is formed and secreted by β-cells of the pancreas. High glucose levels in the blood triggers secretion of insulin which binds to its cellular receptors and promotes cellular glucose utilization. Insulin also reduces blood glucose through stimulating the liver and muscles to generate glycogen (Glucose Metabolism and Regulation: Beyond Insulin and Glucagon), inhibiting glycogenolysis (breakdown of glycogen to release glucose), and preventing gluconeogenesis (synthesis of glucose from non-carbohydrate substrates, largely protein and fat) (Glucose Metabolism and Regulation: Beyond Insulin and Glucagon). In diabetes, changes in the production and secretion of insulin alter blood glucose dynamics. If insulin production is decreased, glucose entry into cells is inhibited, thereby, increasing gluconeogenesis and glycogenolysis resulting in hyperglycaemia (Lin and Accili, 2011), whilst a similar situation is observed with insulin resistance (Zaccardi et al., 2016). Conversely, if insulin secretion is increased, blood glucose levels may become very low (hypoglycaemia) as large amounts of glucose are taken up by cells. Whilst insulin is the only hormone that lowers blood glucose levels, other counter-regulatory hormones, such as glucagon, catecholamines, growth hormone, thyroid hormone, and glucocorticoids act to increase blood glucose levels, in addition to their other metabolic effects (Sprague and Arbeláez, 2011).

1.1.4 Classification of diabetes mellitus
In 1980, the WHO published its original classification of diabetes depending on treatment as insulin–dependent diabetes mellitus (IDDM) or non-insulin dependent diabetes mellitus (NIDDM), and gestational diabetes. In 1985, this was modified to type 1 diabetes, type 2 diabetes and gestational diabetes and adopted widely as it was based on pathogenesis rather than treatment (Alberti and Zimmet, 1998).

1.1.4.1 Type I diabetes mellitus
Type 1 diabetes mellitus (T1DM) accounts for 5-10% of all diabetes cases (American Diabetes Association and Diabetes, 2009) and is sometimes known as autoimmune diabetes. Whilst its insulin-dependent nature was discovered ~100 years ago, the precise mechanisms of disease development are not fully explained, although T cell mediated pancreatic β-cell destruction is known to be involved (Kurrer et al., 1997). Indeed, the pathogenesis of type 1 diabetes is complex, with several immunologic autoantibodies to insulin, glutamic acid decarboxylase or β-islet which are reported to trigger an autoimmune reaction leading to β-cell destruction (Baynest, 2015).
In addition, the function of α-cells in the pancreas can also be abnormal and associated with excessive glucagon secretion. Normally, hyperglycaemia reduces glucagon release, while this is not suppressed in T1DM patients, with the consequent inappropriate glucagon levels exacerbating the metabolic abnormalities due to insulin deficiency (Yosten, 2018). Moreover, insulin deficiency induces uncontrolled lipolysis and high plasma free fatty acid levels which impair peripheral glucose utilization, such as in skeletal muscle. Taken together, these pathological mechanisms result in decreased expression of genes necessary for tissue insulin targeting, such as GLUT 4 in adipose tissue and glucokinase in the liver (Baynest, 2015), with ultimate disruption of glucose, lipid and protein metabolism. The primary therapeutic focus in type 1 diabetes is insulin replacement to achieve normal blood glucose levels, thereby limiting development and progression of microvascular and macrovascular complications which are the major cause of associated morbidity and mortality.

1.1.4.2 Type II diabetes mellitus
Type 2 diabetes mellitus (T2DM) accounts for 90-95% of all diabetic cases (Gutteridge, 1999) and is characterized by relative insulin deficiency or resistance to insulin action, with most affected individuals exhibiting central obesity resulting in insulin resistance (Hu et al., 2002). Other common risk factors are family history, lack of exercise, and older age. Type II diabetes usually goes undiagnosed for years since hyperglycemia develops gradually and may not become severe enough to induce classical symptoms of diabetes (Tamarai, et al, 2019). Therefore, early diagnosis through regular screening programmes, together with effective therapies, is critical to prevent or delay associated complications and reduce resulting morbidity and mortality. In this regard, the goal of T2DM treatment is to achieve HbA1c levels of <7% through lifestyle changes and/or antidiabetic drugs without which initially, these patients do not need exogenous insulin as glycaemia can be controlled by antidiabetic drugs in order to optimise metabolic control and improve patient well-being. (Huri et al, 2015).

1.1.4.3 Gestational diabetes
Gestational diabetes refers to diabetes that develops in women during pregnancy. Women who have a history of diabetes or are overweight are at a higher risk of developing gestational diabetes which may either resolve or develop into overt T2DM upon delivery of the baby. Uncontrolled gestational diabetes may cause mother and baby complications both during pregnancy and later in life, so it is essential that the
condition is effectively diagnosed and managed (Gutteridge, 1999) Diabetes complications

1.1.5 Diabetes complications
A major hallmark of diabetes is increased incidence of macrovascular and microvascular complications (Orasanu and Plutzky, 2009) which may lead to long term damage and eventual failure of organs like the heart, kidneys, eyes, which may be associated with nerve dysfunction (Calcutt et al., 2009) Although intensive glycaemic control can reduce the incidence of diabetes complications, consequent morbidity is still rising (MacIsaac and Jerums, 2011). Landmark large-scale clinical trials, such as the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) demonstrated that strict glycaemic control limits the incidence of microvascular complications, highlighting the importance of glucose control, although data with regard to macrovascular outcomes were not conclusive (Holman et al., 2008).

Several similar large-scale trials have since been performed in the last decade to compare the effect of standard versus intensive glycaemic control on cardiovascular outcomes, namely Action in Diabetes and Vascular disease: Preterax and Diamicron-MR Controlled Evaluation (ADVANCE) and Veterans Affairs Diabetes Trial (VADT). They included type 2 diabetic patients who were at risk of developing cardiovascular events or who had previous history of cardiovascular disease randomised to standard or intensive glycaemic treatment for 3.5-5.6 years. Interestingly, no significant reduction in incidence of progression of cardiovascular outcomes was observed with intensive glycaemic control (Skyler et al., 2009). Indeed, a third trial, Action to Control Cardiovascular Risk in Diabetes (ACCORD), was terminated early due to increased cardiovascular mortality with extensive glycaemic control with a target HbA1C of 6 (Gerstein et al., 2008), indicating that blood glucose is not the only factor mediating diabetes complications.

1.1.5 Macrovacular and microvascular changes in diabetes
Arterioles, venules, and capillaries comprise the microvessels which play a key role in regulating blood pressure and nutrient delivery (Orasanu and Plutzky, 2009), including control of vascular permeability which is critical for adaptation to metabolic needs (Chawla et al, 2016)

Diabetes induces pathological changes in micro-vessel structure including initial thickening of the basement membrane which eventually alters vascular function,
promoting tissue hypoxia, hypertension and impaired wound healing (Chawla et al, 2016). In addition, hypoxia can drive neovascularisation from the vasa vasorum, thereby interconnecting micro and macrovasculopathy which may predict platelet dysfunction and promote atherosclerosis (Tian et al., 2013). Notably, heart disease in diabetes, which is the focus of this thesis, may be driven by both macrovascular atherosclerotic complications and structural microvascular derangement, which together promote development and progression of cardiac dysfunction (Laakso, 2011). However, it is thought that microvascular changes are more important in determining the often-subclinical dysfunction and structural alterations characteristic of diabetic cardiomyopathy (Orasanu and Plutzky, 2010).

1.2 Heart failure and diabetic cardiomyopathy

1.2.1 Correlation between diabetes, cardiovascular disease and heart failure

A strong association between diabetes and cardiovascular diseases has been documented by previous large-scale epidemiological studies. Indeed, people with type 2 diabetes show increased cardiovascular morbidity and mortality compared to non-diabetic patients without previous history of myocardial infarction and carry as high a risk as non-diabetic subjects with previous myocardial infarction. (Haffner et al., 1998). Consequently, the life expectancy of diabetic patients is 5 to 10 years shorter than non-diabetics (Fitchett, 2015), largely due to early development of ischaemic disease, with at least 68% of people age >65 with diabetes dying from myocardial infarction or stroke (Hajar, 2017).

Moreover, the Framingham study demonstrated a 3-fold increased risk of heart failure in diabetic females and a 2-fold increase risk in male patients with diabetes compared with nondiabetics counterparts after adjustment for other risk factors including age, obesity, hypertension, coronary artery disease (Kannel and Mc Gee, 1979). Whilst atherosclerotic diseases, such as myocardial infarction and stroke are considered the predominant cardiovascular disease manifestations in T2D. Data from the UK National Diabetes Audit 2015–2016, including 2.7M diabetic patients, reported heart failure (HF) as the most common cardiovascular complication of Type 2 diabetes and major cause of premature mortality (Gulsin et al, 2019). Notably, although the risk of death due to stroke and myocardial infarction may be alleviated with cardiovascular risk factor management, heart failure risk persists despite proper cardiovascular risk control (Rawshani et al., 2018). Moreover, the co-existence of diabetes in patients
with stable chronic HF is responsible for a three-fold higher rate of hospitalisation for patients with decompensated ventricular dysfunction (Cubbon et al., 2014).

The Reduction of Atherothrombosis for Continued Health (REACH) registry, an international database of patients with established atherothrombosis or at high risk of atherothrombosis, investigated long-term cardiovascular outcomes in diabetic patients. At 4 years, it reported 33% higher risk of hospitalization due to heart failure (9.4% versus 5.9%; adjusted odds ratio, 1.33; 95% CI, 1.18–1.50) and independent association of HF with cardiovascular death (adjusted HR, 2.45; 95% CI, 2.17-2.77; P<0.001) in diabetic patients (Cavender et al., 2015). Similarly, the Candesartan in Heart failure - Assessment of Reduction in Mortality and morbidity (CHARM) trial, which recruited 7599 symptomatic heart failure patients with broad range of ejection fraction, indicated that diabetes is an independent predictor of cardiovascular morbidity and mortality in patients with heart failure regardless of the ejection fraction (MacDonald et al., 2008). In addition, diabetic patients demonstrate increased echocardiographic left ventricular wall thickness (Devereux et al., 2000) and poor response to optimal diuretic therapy and heart failure pharmacotherapy compared to non-diabetic individuals (Nasir and Aguilar, 2012). Notably, many trials have reported correlation of what has been described as the cardiotoxic triad in heart failure: hypertension, diabetic cardiomyopathy, and myocardial ischaemia, which seem to cooperatively contribute to structural, functional and biochemical changes in the heart leading to maladaptive remodelling and dysfunction (Bell, 2003).

1.2.2 Heart failure
The American Heart Association/American College of Cardiology guidelines define heart failure as inability of the heart to pump enough oxygenated blood to meet body tissues demands. It is a clinical syndrome that develops due to functional or structural impairment of ventricular filling or ejection which is characterised by symptoms of congestion, such as peripheral oedema, dyspnoea and fatigue (Hunt et al., 2005). This definition comprises a broad spectrum of myocardial dysfunction manifestations including diastolic or systolic ventricular dysfunction or both in the left side or right side or both and cardiac valvular disease.

1.2.2.1 Epidemiology of heart failure
Heart failure is a worldwide public health problem which affects at least 26M people (Ambrosy et al., 2014). In the US alone, it is predicted that more than 8M people will have the disease by 2030, representing a 46% increase in HF prevalence (Benjamin
et al., 2019). Significantly, ~74% of heart failure patients suffer from at least one co-morbidity, such as diabetes or anaemia, which are more likely to worsen the patient’s overall quality of life and chances of survival (Van Deursen et al., 2014). Hospitalization of patients with heart failure is associated with high rates of post-discharge mortality and readmission (Gheorghiade et al., 2013), which have reported as ~25% within 30 days with all-cause mortality of >30% at 1-year (Marti et al., 2013). In addition, HF represents a major economic burden, which in the US was estimated at $31 billion in 2012, rising to $70 billion by 2030 (Heidenreich et al., 2013).

1.2.2.2 Aetiology of heart failure
The causes of HF can be broadly divided into cardiac and systemic. Cardiac causes include conditions resulting in reduced filling reduction in which diastolic relaxation is impaired due to hypertrophic cardiomyopathy, and fibrosis or structural abnormalities, such as constrictive pericarditis or valvular disease (Mosterd and Hoes, 2007). Other cardiac causes include reduced ejection fraction due to reduced systolic function due to, for example, ischaemia or aortic obstruction/coarctation (Chatterjee and Rame, 2008). Systemic causes such as hypertension, kidney disease, diabetes mellitus (Ziaeian and Fonarow, 2016), and anaemia may both induce and aggravate HF (Wexler et al., 2005; Mosterd and Hoes, 2007; Pearse and Cowie, 2014). Diabetes (particularly type 2) is considered as a major risk for heart failure (Bertoni et al., 2004). Despite adjustment for well-established risk factors, such as ischaemic heart disease, valvular disease or hypertension, it has been suggested that diabetes-associated hyperglycaemia may adversely alter cardiac structure and function, a condition often referred to as diabetic cardiomyopathy (Bugger and Abel, 2014). Pure diabetic cardiomyopathy was first described almost 50 years ago (Rubler et al., 1972) and is defined as cardiac dysfunction in the absence of coronary artery disease, and is proposed to drive left ventricular remodelling and dysfunction, ultimately contributing to heart failure development (Bugger and Abel, 2014).

1.2.2.3 Physiological response to heart failure
Neurohumoral responses to compensate for insufficient cardiac output result largely comprise activation of renin-angiotensin-aldosterone system (RAAS) and sympathetic nervous system. RAAS activation in response to reduced renal perfusion promotes vasoconstriction and fluid retention, mediated by angiotensin II and aldosterone, respectively, whilst β1-adrenergic receptor stimulation, due to reduced cardiac output, increases heart rate (chronotropy) and contractility (inotropy). Although both of these mechanisms initially serve to improve cardiac output, these
changes eventually become maladaptive as the heart is no longer able to compensate for prolonged stress (Hartupee and Mann, 2017). Chronically elevated cardiac workload may result in cardiomyocyte apoptosis and necrosis, due to limited oxygen supply, and hypertrophy and remodelling associated with fibrosis and structural changes, due to haemodynamic stress, leading to cardiomyopathy and systolic/diastolic dysfunction (Piek, et al, 2016). In addition, the cardiomyocytes undergo complex membrane protein changes that alter intracellular calcium signalling (Bers and Despa, 2006), which combined with aberrant fibrosis may predispose to cardiac arrhythmia (Fenton and Burch, 2007).

1.2.2.4 Stages and management of heart failure
HF is classified in stages as mentioned in Figure 1.1. Based on cardiac structural and functional abnormalities (ACA/American Heart Association; AHA) from A to D in which stage A: asymptomatic HF, stage B: structural cardiac changes with no symptoms of HF, stage C: clinical manifestation of HF, stage D: end-stage or refractory HF. Alternatively, it can be classified according to symptoms and physical capability (New York Heart Association; NYHA) as class I: no limitation in physical capability, class II: slight limitation in which ordinary physical activity results in HF symptoms, class III: Marked limitation on physical activity, and class IV: unable to execute any physical activity without development of heart failure symptoms (Inamdar and Inamdar, 2016; Lam, 2015).

HF management initially targets contributing factors, lifestyle modification, and pharmacological therapy with the classification of HF stages A to D guiding clinicians to initiate and titrate the medication dose (Figure 1.2). Medications targeted at modifying the underlying neurohumoral pathophysiological mechanisms have been considered as the basis of HF care for several decades, specifically targeting preload, afterload and/or cardiac contractility.
Diuretics are often the first-line treatment for HF and are essential to overcome symptoms of fluid overload, whilst positive inotropic agents and vasodilators may promote cardiac contractility and vasodilatation, thereby reducing workload. Angiotensin-converting enzyme inhibitors (ACEIs), angiotensin-receptor blockers (ARBs), aldosterone antagonists and β-blockers either block the effects or inhibit the production of neurohormones contributing to HF progression (Gordin and Fonarow, 2016). Together, these medications are reasonably effective in managing heart failure symptoms and improving cardiac function. However, in cases where medical treatment is partially effective, cardioverter defibrillators and cardiac resynchronization therapy may be used for advanced heart failure.

Regardless of optimal clinical management, there remains a high morbidity and mortality associated with heart failure which requires the development of new treatment strategies (Janaswamy et al., 2016).

<table>
<thead>
<tr>
<th>ACA/AHA stages of heart failure (Based on structure and function)</th>
<th>NYHA classification of heart failure (Based on symptoms and physical capability)</th>
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<tbody>
<tr>
<td><strong>Stage A</strong></td>
<td>Patients at high risk of developing HF because of the presence of conditions that are strongly associated with the development of HF. Such patients have no identified structural or functional abnormalities of the pericardium, myocardium, or cardiac valves and have never shown signs or symptoms of HF.</td>
</tr>
<tr>
<td><strong>Stage B</strong></td>
<td>Patients who have developed structural heart disease that is strongly associated with the development of HF but who have never shown signs or symptoms of HF.</td>
</tr>
<tr>
<td><strong>Stage C</strong></td>
<td>Patients who have current or prior symptoms of HF associated with underlying structural heart disease.</td>
</tr>
<tr>
<td><strong>Stage D</strong></td>
<td>Patients with advanced structural heart disease and marked symptoms of HF at rest despite maximal medical therapy and who require specialized interventions.</td>
</tr>
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Figure 1-1 ACA/AHA stages and NYHA classification of heart failure based on structure/function and symptoms/physical capability. Adapted from Heart Failure Society of America (2011; 2001)
1.2.3 Diabetic cardiomyopathy

1.2.3.1 Definition of diabetic cardiomyopathy

Almost fifty years ago, diabetic cardiomyopathy was first described by Rubler and colleagues based on post-mortem data from four diabetic patients who died from heart failure without evidence of hypertension, valvular disease or cardiac ischaemia (Rubler et al., 1972). They specifically observed the presence of myocardial fibrosis associated with left ventricular hypertrophy in the absence of coronary artery disease (Rubler et al., 1972), results confirmed five years later by Regan et al (1977) who reported higher cardiac collagen and lipid accumulation in diabetic patients compared with control subjects (Regan et al, 1977). In addition, a more recent study demonstrated increased risk of heart failure in type 2 diabetic patients’ independent of associated cardiovascular etiological factors such as hypertension and myocardial infarction (Bugger and Abel, 2014), supporting existence of distinct diabetic cardiomyopathy.

This has been defined as the existence of LV dysfunction in diabetic patients without hypertension, coronary artery disease (CAD), or other potential risk conditions, with both clinical and experimental studies reporting early left ventricular hypertrophy and diastolic dysfunction, progressing to systolic dysfunction and HF (Schilling and Mann, 2003).
 Nonetheless, the existence of pure diabetic cardiomyopathy remains controversial with many arguing that diabetic cardiomyopathy is present in diabetic patients in parallel with other cardiac risk factors. Although it is difficult to assess the precise contribution of diabetic cardiomyopathy to ventricular dysfunction and overt HF. It is clear that diabetic heart is associated with distinct functional and remodelling changes which precede HF development.

1.2.3.2 Metabolic and structural changes in diabetic cardiomyopathy
It is well established that diabetes is associated with left ventricular diastolic dysfunction which may progress to systolic dysfunction, in parallel with cardiomyocyte hypertrophy, increased apoptosis, interstitial fibrosis and increased oxidative stress. (Borghetti et al., 2018). These changes are complex and involve multiple interrelated underlying mechanisms, which are summarised in Figure 1.3. Systemic metabolic changes in diabetes, such as hyperglycaemia, insulin resistance, reactive oxygen species generation, increased circulating triglycerides and inflammatory cytokines may promote altered cardiomyocyte calcium handling, structure/function, and necrosis (Zhang and Chen, 2012; Mandavia et al., 2013), which contribute to development of diastolic dysfunction linked with interstitial fibrosis, adverse cardiac remodelling, and subsequent systolic dysfunction (Zhang and Chen, 2012; Mandavia et al., 2013).
The Strong Heart Study specifically linked severity of diastolic dysfunction with HbA1c levels (Liu et al., 2001b), whilst cardiac diastolic changes have been reported independently of hypertension and coronary artery disease. (Kasznicki and Drzewoski, 2014). In addition, established experimental models of both type 1 and type 2 diabetes display echocardiographic diastolic dysfunction (Schilling and Mann, 2012). The most likely mechanism underlying diastolic dysfunction in diabetes appears to be hyperglycaemia-induced advanced glycation end-products (AGEs) generation and formation of reactive oxygen species (ROS), leading to myocardial collagen deposition and left ventricular hypertrophy (Kasznicki and Drzewoski, 2014).

Diastolic dysfunction can be detected by conventional Doppler echocardiography, based on indices such as isovolumetric ventricular relaxation time (IVRT) (Satpathy et al., 2006). Diastolic function is typically measured by peak blood flow velocity through the mitral valve to assess myocardial filling and left ventricular relaxation using a ratio of initial early peak (E, early) to late (A, atrial) blood flow velocity (Figure 1.4). Prolonged IVRT coupled with reduced E/A ratio are prominent manifestations of diastolic dysfunction observed in diabetic patients (You Fang et al., 2003). As well, it
can be detected by tissue Doppler imaging as the ratio of conventional early (E) flow velocity to diastolic early (E’) tissue (annulus) velocity where the (E/E’) ratio is impaired in diabetic patients (Kim et al., 2013).

1.2.3.2 Pathological changes in the diabetic heart
Several hypotheses underlying pathogenesis in diabetic heart have been proposed, including autonomic dysfunction, abnormalities in ion homeostasis and modulation of structural proteins. Indeed, diabetic heart biopsies have shown increased advanced glycation end-products (AGEs), which are known to initiate inflammatory signalling and myocardial immune cell infiltration, leading to interstitial fibrosis and cardiomyocyte hypertrophy (Tziakas et al, 2005), and consequent myocardial stiffness, reduced diastolic compliance, and heart failure with reduced ejection fraction (Van Heerebeek et al., 2008).

1.2.3.3 Characteristic features of the diabetic heart
It is widely accepted from both experimental and clinical evidence that the diabetic heart is associated with distinct structural and functional changes. At the cellular level, major mechanisms that promote cardiac remodelling involve both cardiomyocytes and non-cardiomyocytes. Loss of cardiomyocytes may occur through apoptosis and
necrosis, while the diabetic heart is characterised by disproportional fibrosis driven by fibroblast differentiation, proliferation and extracellular matrix (ECM) reorganisation (Schirone et al., 2017). Consequently, typical cardiac anatomical and structural changes develop in diabetes which are characterised by myocardial hypertrophy, fibrosis, apoptosis and aberrant ECM turnover, which together contribute to the complex and multifactorial biology underlying cardiac remodelling in diabetes (Schirone et al., 2017).

1.2.3.3.1 Cardiac hypertrophy

Left ventricular hypertrophy (LVH) is considered as a hallmark of diabetic heart, as revealed by increased echocardiographic LV wall thickness and mass, whilst the right ventricle may also become hypertrophied (Huynh et al., 2012). Left ventricular hypertrophy develops as an adaptive response to reduced numbers of functional cardiomyocytes due to cell death consequent of decreased vascular density (Izumiya et al., 2006), and increased hemodynamic stress caused by hypertension and neurohumoral stimulation (Eguchi et al., 2008) (Figure 1.5).

The mechanisms underlying LVH in diabetes are complex, with experimental studies suggested that hyperinsulinemia serves to increase cardiomyocyte mass due to its function as a growth factor. Indeed, mice with genetic deletion of insulin receptors demonstrate reduced cardiac mass (Pires et al., 2017), whilst clinical studies have reported increased insulin receptor activation in cardiac biopsies of type 2 diabetic patients (Cook et al., 2010). In addition, hyperglycaemia and RAAS activation are known to play a key role in determining cardiac hypertrophy in diabetes (Essick and Sam, 2011), with targeting of increased ROS generation shown to reduce cardiac hypertrophy associated with experimental type 1 diabetes, as indicated by decreased expression of hypertrophic genes, such as atrial natriuretic peptide (ANP), β-myosin heavy chain, and brain natriuretic peptide (BNP) (Huynh et al., 2013), which is considered as a prognostic marker of heart failure in diabetic patients (van der Horst et al., 2010).

1.2.3.3.2 Cardiac necrosis and apoptosis

In addition to cardiomyocyte hypertrophy, cell death induced by necrosis and/or apoptosis is a pathological manifestation of diabetic cardiomyopathy which has been described in both patients and animal models (Chowdhry et al, 2007). Cardiomyocyte necrosis refers to cell destruction due to chemical or mechanical trauma, as evaluated by DNA double strand damage, whereas cardiomyocyte apoptosis refers to the
process of controlled cell destruction to remove unwanted or damaged cells, which can be identified by DNA cleavage (Fang et al, 2004). Although the percentage of apoptotic cardiomyocytes in the failing heart is low, it is 10- to 100-fold higher than that observed in control hearts, suggesting that apoptotic loss of cardiomyocytes promotes heart failure development and progression (Moe and Marín-García, 2016).

In fact, the several mechanisms relevant to diabetes have been attributed to increased cell death, including hyperglycaemia-induced mitochondrial ROS generation (Varma et al., 2012), and activation of RAAS, which are correlated with increased cardiomyocyte and endothelial cell apoptosis and necrosis in the hearts of diabetic patients. In this regard, inhibition of RAAS is reported to reduce cell death whilst improving patient cardiac echocardiographic indices and decreasing hospitalisation (Kasznicki and Drzewoski, 2014).

Figure 1-5: Overview of cardiomyocyte and non-cardiomyocyte changes during cardiac remodelling. Different pathological signalling promotes cardiomyocyte (CM) loss and myofibroblast differentiation, leading to cardiac hypertrophy and fibrosis, changes in cardiac geometry, and ultimately heart failure. Adapted from (Schirone et al., 2017)

In addition, increased inflammation, which is a characteristic feature of diabetes, has been shown to mediate proapoptotic activation of caspase-3 in mouse hearts, thereby
accelerating cardiomyocyte and endothelial cell death and cardiac functional
impairment (Cai et al., 2002).

It is important to note that apoptosis and necrosis, as two distinct forms of cell death, result in different consequences. Whilst apoptosis does not cause scar formation because vacated tissue space is replaced by the surrounding cells, cellular necrosis promotes increased extracellular matrix remodelling (Eckhouse and Spinale, 2012). Specifically, diabetes-induced cardiomyocyte apoptosis is often associated with increased cardiac collagen deposition and interstitial fibrosis, thereby contributing to the characteristic phenotype of diabetic cardiomyopathy.

1.2.3.3 Fibrosis
Myocardial fibrosis has been largely implicated in diabetic cardiomyopathy and is at least partly attributed to replacement of apoptotic cardiomyocytes by fibrous tissue. Importantly, both perivascular and interstitial fibrosis are observed in diabetic hearts of both humans and animals in the absence of hypertension or coronary artery disease (Shimizu et al., 1993), highlighting aberrant ECM remodelling as a causative factor in subsequent heart failure development. For example, hearts of STZ-induced type 1 diabetic rats and Otsuka Long Evans Tokushima fatty (OLETF) type 2 diabetic rats, both show increased collagen accumulation associated with myocardial stiffness and impaired ventricular relaxation (Lee et al., 2012a; Wu et al., 2017). Similarly, heart biopsies from diabetic patients show increased perivascular and interstitial cardiac fibrosis (Regan et al., 1977), with specific elevation of collagen types I, III and VI in the myocardial interstitium compared to non-diabetic controls (Shimizu et al., 1993). Indeed, cardiac diastolic dysfunction in type 2 diabetic patients is correlated with levels of procollagen type I carboxy-terminal peptide (Ihm et al., 2007), supporting direct association between myocardial fibrosis and dysfunction.

The pathological mechanisms underlying cardiac fibrosis are complex and may include excess accumulation of collagen, reduced extracellular matrix degradation, and/or chemical modification of ECM proteins. Specifically, these may involve increased expression of transforming growth factor beta (TGF-β) and connective tissue growth factor (CTGF), leading to enhanced collagen type I and type III and ECM crosslinking (Segura et al, 2014). Formation of advanced glycation end-products (AGEs) in diabetes promotes cardiac fibrosis and ECM remodelling by activation of oxidative stress pathways, RAAS (Matsusaka et al., 2006) and pro-inflammatory signalling (Russo and Frangogiannis, 2016). AGEs also increase
collagen resistance to proteolysis whilst reducing degradation by matrix metalloproteinases (MMPs) (Aronson, 2003). In combination, these ECM alterations reduce cardiac elasticity and disturb electrical conductance leading to inappropriate contractility, cardiac arrhythmias and sudden death (Russo and Frangogiannis, 2016).

Ultimately, these pathological mechanisms are a major contributor to impaired left ventricular diastolic function observed in diabetic patients. Notably, hyperglycaemia alone is sufficient to activate these profibrotic pathways, thus promoting myocardial fibrosis and cardiac dysfunction. In this regard, it is important to highlight that tight blood glucose control, inhibition of RAAS, prevention of oxidative stress and suppression of inflammation can improve cardiomyocyte and endothelial cell survival, and prevent fibroblasts differentiation, thereby preserving left ventricular function in diabetic patients.

1.2.3.4 ECM remodelling

Ventricular remodelling in diabetes is associated with accumulation of collagen between cardiomyocytes and changes in ECM protein expression. The ECM maintains normal tissue architecture and homeostasis (Valiente-Alandi et al, 2016) and is mainly composed of collagen type I and type III, produced by fibroblasts, and the basement membrane comprising fibronectin, collagen IV, fibrillin and laminin. (Brower et al., 2006). This structural arrangement is important to support physiological cardiac structure, shape and recoil during diastole and to prevent overstretching of cardiomyocytes (Moore et al., 2012). As a result, disruption of this ECM network reduces cardiomyocyte structural support leading to changes in myocardial geometry and function. Pathological cardiac remodelling in diabetes is characterised by excessive accumulation of ECM proteins leading to abnormal cardiac architecture and dysfunction (Travers et al., 2016).

Hyperglycaemia and other metabolic pathways like angiotensin II and endothelin-1 induce profibrotic signalling thus activating proinflammatory cytokines and chemokines and promoting cardiac leukocyte infiltration (Russo and Frangogiannis, 2016). Myocardial inflammation drives structural and functional ECM changes, resulting in reduced cardiac repair, maladaptive cardiac remodelling, and heart failure progression. Specifically, ECM remodelling results from two concurrent processes: increased synthesis of ECM structural proteins, mainly collagen, and dysregulation of proteolytic enzymes, matrix metalloproteinases (MMPs) and tissue inhibitors of
metalloproteinases (TIMPs), a balance between which plays a major role in controlling normal ECM homeostasis (Figure 1.6) (Moore et al., 2012).

MMPs are zinc dependent proteases, synthesised as inactive pro-MMPs, which are activated by removal of the amino-terminal of the propeptide domain (Spinale, 2002), and comprising collagenases, and gelatinases (Nagase et al, 2006). MMPs are activated by proinflammatory cytokines, such as TNF-α and IL-1, and a variety of growth factors and hormones. For example, TNF-α increases the secretion of MMP-9 and MMP-13 from cardiac fibroblasts (Siwik et al, 2000), whilst altering the MMP:TIMP ratio, thereby promoting increased cardiac collagen content and LV structural remodelling, as reported in TNF-α transgenic mice (Sivasubramanian et al., 2001). Similarly, transforming growth factor-β (TGF-β) is an important regulator of MMP gene expression, by specifically increasing TIMP expression which reduces proteolytic activity of MMPs (Li et al, 2000). In this regard, activated MMPs can be reversibly inhibited by TIMPs via formation of a covalent 1:1 complex, occurring through interaction between the MMP-Zn$^{2+}$ active site and terminal cysteine residue of TIMPs (Moore et al., 2012). Indeed, downregulation of TIMPs has been reported in the failing heart in parallel with upregulation of MMP-9, which is known to drive pathological ventricular remodelling in HF (Li et al., 1998). Taken together, imbalance between MMP and TIMP activity/expression in the failing heart favours persistent MMP activation, ECM proteolysis, and adverse myocardial remodelling.

As such, MMP inhibition is considered as a significant potential therapeutic strategy for alleviation of maladaptive LV remodelling and heart failure progression.
Figure 1-6 The role of MMPs and TIMPs in cardiac remodelling. Fibroblasts are differentiated by TGF-β to myofibroblasts which secrete collagen. The ECM undergoes remodelling further to the opposite actions of MMPs and TIMPs – MMPs degrade collagen, whilst TIMPs inhibit MMPs. MMPs: Matrix metalloproteinases; TIMPs: Tissue inhibitors of metalloproteinases

1.2.3.4 Pathophysiological triggers of diabetic cardiomyopathy

1.2.3.4.1 Hyperglycaemia

The mechanisms underlying development of diabetic cardiomyopathy are complex but interrelated with hyperglycaemia considered as the main pathophysiological driver. Aberrant glucose control and/or insulin secretion represent the main factors eliciting molecular and cellular changes via generation of ROS and AGEs (Wilson et al., 2018), and fatty acid oxidation (Lopaschuk et al., 2010).

AGEs are specifically induced by hyperglycaemia-mediated glycosylation of proteins and lipids, leading to increased collagen crosslinking and reduced degradation ability, thus promoting fibrosis, myocardial stiffness and consequent prolongation of left ventricular relaxation (Norton et al., 1996; Singh et al., 2001). Indeed, aminoguanidine, which inhibits AGE formation, is reported to alleviate cardiac and vascular dysfunction in streptozotocin (STZ)-induced type 1 diabetic rats (van Niekerk, 2008).

AGEs trigger biological responses through binding to their receptors (RAGEs), which are members of an immunoglobulin superfamily expressed on cardiomyocytes, macrophages, smooth muscle cells and endothelial cells (Ramasamy et al., 2011). Binding of AGEs to RAGE activates nuclear factor-κB (NF-κB) (Jia et al., 2016) and NADPH oxidase signalling, triggering production of inflammatory cytokines and ROS, respectively (Aragno et al., 2006), which can be inhibited by dehydroepiandrosterone, thus reducing cardiac collagen I, collagen IV, and fibronectin levels and consequent
cardiomyopathy in both streptozotocin-induced T1DM rats and Zucker diabetic fatty (ZDF) T2DM rats (Aragno et al., 2006). Interestingly, administration of a RAGE antagonist to type 1 diabetic mice is reported to prevent AGE/RAGE-mediated myocardial collagen accumulation, fibrosis, and diastolic dysfunction (Ma et al., 2009). Therefore, as hyperglycaemia associated activation of AGE/RAGE signalling is a significant contributor to cardiac fibrosis and diastolic dysfunction, therapeutic targeting of this pathway may be beneficial in preventing diabetic cardiomyopathy.

Hyperglycaemia and insulin resistance cause alterations in cardiomyocyte calcium handling and contractility, as reported in several experimental models of T1DM and T2DM. This is mainly mediated by modulation of expression and function of endoplasmic/sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) and other transporters leading to impaired calcium haemostasis, abnormal calcium handling, and dysfunctional excitation-contraction coupling (Sweedner and Donnet, 2001). Normal cardiac relaxation is mediated by SERCA-regulated movement of calcium ions from the cytosol into the sarcoplasmic reticulum, and Ca\(^{2+}\) expulsion via sarcolemmal Na\(^+\)-Ca\(^{2+}\) exchange (NCX) and the plasma-membrane Ca\(^{2+}\) ATPase (PMCA) (Bers and Despa, 2013). However, in diabetes, hyperglycaemia-associated ER stress impairs calcium reuptake and increases action potential duration leading to slowing of diastolic relaxation and cardiac dysfunction (Poornima et al, 2006). In diabetic rats, crosslinking of AGEs to SERCA-Ca is reported to alter sarcoplasmic reticulum (SR) Ca\(^{2+}\) reuptake in cardiomyocytes leading to impaired cardiac function (Bidasee et al., 2004), which can be partially reversed by breaking AGE crosslinks (Kranstuber et al., 2012).

AGEs may also impact on cardiomyocyte contractility via increasing oxidative stress and ROS production, which also induce inflammatory cytokines and angiotensin II-mediated cardiac fibrosis (Aragno et al., 2006). Indeed, experimental type 1 and type 2 diabetes accentuates the effects of oxidative stress in cardiac remodelling. For example, in T2DM db/db mouse hearts perfused with glucose, mitochondrial ROS generation is increased in association with uncoupling protein and lipids peroxidation (Boudina et al., 2007), resulting in unfavourable cardiac efficiency and low ATP proliferation. Moreover, increased lipid peroxidation and glutathione oxidation observed in hearts of streptozotocin-induced T1DM rats has been shown to involve activation of xanthine oxidase (Desco et al., 2002). Indeed, under physiological conditions, most cellular ROS are generated by mitochondria (Chance et al, 1979), whilst other ROS sources may emerge under pathological conditions. (Elahi et al,
In this regard, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes are recognised as a major source of ROS, through catalysing electron transfer from NADPH to molecular oxygen, inducing free radical generation (Selemidis et al., 2008). If ROS production exceeds degradation by endogenous defence antioxidants, such as glutathione peroxidase and manganese superoxide dismutase (Seddon et al, 2007), that may increase ROS levels to induce further mitochondrial dysfunction which promotes harmful effects on the cardiovascular system via initiation of cellular damage, cardiomyocyte apoptosis, modulation of intracellular signalling, disruption of cardiovascular nitric oxide (NO) homeostasis, and altered cardiac contractile function (Verma et al, 2017).

1.2.3.4.2 Impaired myocardial insulin signalling
Diabetic cardiomyopathy associated with insulin-resistance or hyperinsulinemia is characterised by impaired myocardial insulin signalling, linked with endoplasmic reticulum stress, mitochondrial dysfunction, and RAAS activation (Jia et al, 2018), which contribute to pathophysiological changes in cardiac fibrosis, hypertrophy, diastolic/systolic dysfunction and heart failure. Under normal physiological conditions, insulin stimulates tissue glucose uptake to maintain metabolic homeostasis, whilst impaired insulin signalling and/or insulin resistance in diabetes is associated with reduced glucose transport which eventually progresses to hyperinsulinaemia (Adeghate and Singh, 2014). As such, insulin resistance and/or hyperinsulinaemia are often associated with cardiac metabolic disorders including diabetes and contribute to the early stages of cardiovascular disease development.

Insulin signalling occurs through two key cellular pathways. The first pathway promotes increased phosphorylation of insulin receptor substrate 1 (IRS-1) and downstream activation of phosphatidylinositol 3-kinase (PI3K)–protein kinase B (also known as AKT) signalling which induces translocation of GLUT4 to cardiac cell membrane thereby increasing glucose uptake (Jia et al, 2016). Notably, PI3K can also increase nitric oxide production through activation of nitric oxide synthase, thus promoting coronary artery vasodilatation, substrate flexibility and energy homeostasis, whilst RAAS stimulation or excessive nutrient uptake drives the mammalian target of rapamycin (mTOR)–S6 kinase 1 (S6K1) pathway (Kim et al., 2012). This, in turn, attenuates insulin-dependent IRS-1/PI3K/AKT signalling in cardiac tissue, thereby promoting insulin resistance and reduced myocardial glucose uptake, and subsequently decreased Ca^{2+} ATPase activity and sarcoplasmic reticulum Ca^{2+} uptake, leading to increased intracellular Ca^{2+} (Jia et al, 2016).
addition, impaired cardiac glucose uptake may inhibit endothelial nitric oxide-mediated coronary vascular relaxation (Aroor et al, 2012), further driving cardiac hypertrophy, impaired myocardial-endothelial signalling, fibrosis, and diastolic dysfunction (King et al, 2016). Consequently, cardiac insulin resistance associated with metabolic and functional abnormalities in diabetes often progress to overt cardiomyopathy.

The second insulin pathway involves mitogen activated protein kinase (MAPK) which enhances expression of endothelin-1 (ET-1) and plasminogen activator inhibitor-1 (PA-1), and encourages proliferation of vascular cells which promote atherosclerosis and vasoconstriction (King et al, 2016). As this pathway is no longer suppressed in diabetes, due either to hyperglycaemia or hyperlipidaemia, these detrimental actions of insulin signalling may also contribute to adverse cardiovascular structural and functional remodelling. Thus, the insulin-resistant state, often observed in diabetes, favours harmful proatherogenic AMPK signalling rather than cardioprotective antiatherogenic IRS-1/PI3K/AKT signalling (Jia et al, 2016).

1.2.3.4.3 Inappropriate Renin-Angiotensin-Aldosterone System activation
The key role of the renin-angiotensin-aldosterone system (RAAS) in cardiac remodelling is well established (Boudina and Abel, 2007). Expression of angiotensin II and the angiotensin II receptor 1 (AT1) is increased in experimental diabetes and important in driving associated diabetic cardiomyopathy (Miller, 1999; Reuter et al., 2006). Furthermore, intracellular concentrations of angiotensin II are reported as 3.4-fold higher in cardiomyocytes from diabetic patients compared with non-diabetic controls (Frustaci et al., 2000). In addition, activation of both AT1 and mineralocorticoid receptor signalling by angiotensin and aldosterone respectively induces coronary endothelial leukocyte adhesion, myocardial macrophage infiltration and polarization towards a proinflammatory M1 phenotype, together with increased proinflammatory cytokine expression, which exacerbates maladaptive cardiac interstitial fibrosis, remodelling and diastolic dysfunction (Jia et al., 2015b).

Notably, secretion of both angiotensin II and aldosterone in diabetes promotes NADPH oxidase-induced ROS generation, contributing to the characteristic cardiac structural and functional phenotype (Privratsky et al., 2003). Indeed, attenuation of RAAS signalling by angiotensin-converting enzyme (ACE) inhibition is reported to reduce ROS production in streptozotocin diabetic rats, effects also observed with exogenous antioxidant treatment (Fiordaliso et al., 2006), confirming the specific role of RAAS-induced ROS generation in this setting. Similarly, another study using the
ACE inhibitor, captopril in diabetic rats demonstrated partial prevention of cardiac interstitial and perivascular fibrosis and increased capillary perfusion (Rösen et al., 1995). Importantly, clinical trials have also indicated reduced cardiovascular remodelling and cardiac mortality in heart failure patients treated with the aldosterone antagonist, spironalactone (Zannad et al., 2000). Taken together, these observations are compatible with the assumption that RAAS activation promotes cardiac remodelling in diabetes and that treatment with ACE inhibitors or aldosterone antagonists may promote cardioprotective effects in diabetic patients.

1.2.3.4.4 Cardiac lipotoxicity
The heart is capable of utilising diverse substrates for ATP generation. Indeed, substrate flexibility is a hallmark of cardiac metabolism that serves to protect cardiomyocytes from injury in situations of decreased substrate availability and/or increased ATP demand (Karwi et al., 2018). The diabetic myocardium is specifically characterised by reduced glucose utilisation (both glucose uptake and oxidation) and elevated fatty acid metabolism (Bugger and Abel, 2014), as reported by both human and animal studies, with this substrate switch representing a key contributor to cardiomyopathy development (Lopaschuk, 2002).

In addition, whilst increased triacylglycerol levels in diabetes lead to increased CD36-mediated fatty acid (FA) uptake by cardiomyocytes, this may exceed the maximum capacity for β-oxidation, thereby resulting in cardiac lipid deposition, energy deprivation, and worsening insulin resistance, and promotion of cardiomyopathy (Sharma et al., 2004; Jia et al., 2018). One of the most significant metabolic changes in the diabetic heart underlying substrate switching is increased expression of peroxisome proliferator-activated receptor-α (PPAR-α) (Finck et al., 2002), which promotes FAs uptake and oxidation whilst suppressing glucose utilisation (Lee et al., 2017).

Increased lipid metabolites generated by the diabetic myocardium may also impair insulin signalling, further driving cardiomyopathy. One such example is diacylglycerol which impairs cardiomyocyte glucose metabolism by activation of protein kinase C, thereby increasing insulin resistance and reducing NO production (Atkinson et al., 2003). Another lipid metabolite is ceramide, a sphingolipid that can trigger cardiomyocyte apoptosis through attenuation of GLUT4-mediated glucose transport and increased oxidative stress (Atkinson et al., 2003). In addition to these metabolic effects, cardiac fatty acid accumulation enhances opening of Na⁺/K⁺-ATP channels.
leading to action potential shortening and depleted sarcoplasmic reticulum calcium stores, thus diminishing cardiomyocyte contractility (Liu et al., 2001a). As such, increased fatty acid uptake in the diabetic heart serves to stimulate lipid metabolite generation, increase mitochondrial ROS formation, and alter cardiac calcium stores, which together significantly contribute to the pathogenesis of diabetic cardiomyopathy.

1.2.3.4.5 Maladaptive immune modulation
It has been reported in various mouse models of diabetes that metabolic disturbances, such as insulin resistance and hyperglycaemia, activate multiple inflammatory signalling pathways that contribute to cardiac remodelling (Bugger and Abel, 2014). For example, AGE/RAGE signalling promotes myocardial inflammation through activation of NF-κB and downstream induction of proinflammatory cytokines, including TNFα, IL-6, pro IL-1, and monocyte chemotactic protein 1 (MCP-1), which drive cardiac oxidative stress and diastolic dysfunction (Fuentes-Antrás et al., 2014). Indeed, myocardial inflammation in association with increased TNFα and IL-1 expression in infiltrating cells has been reported in STZ-induced diabetic rats, in which TNFα inhibition attenuated adhesion molecule expression (ICAM-1, VCAM-1) in parallel with reduced cardiac fibrosis and improved LV function (Westermann et al., 2007). NF-κB also promotes activation of the myocardial pyrin domain-containing 3 (NLRP3) inflammasome (Luo et al., 2014), which plays a crucial role in diabetic cardiomyopathy development. Therefore, NLRP3 gene silencing is reported to suppress cardiomyocyte inflammation by inhibiting release of TNF-α, IL-1β, IL-6, and IL-18. Increased infiltration of inflammatory cells, such as T-lymphocytes and macrophages, is recognised as a significant contributor to myocardial inflammation in diabetes and subsequent maladaptive remodelling (Jia et al., 2015a; Tate et al., 2016). Indeed, proinflammatory M1 macrophage polarisation is predominant in the diabetic heart, while anti-inflammatory M2 macrophage polarisation is suppressed (Urbina and Singla, 2014). In this regard, M1 macrophages (F4/80+, CD11c+) secrete inflammatory cytokines associated with cardiac fibrosis (Jia et al, 2016), whilst M2 macrophages (F4/80+, CD206+, CD11c−) secrete IL-10 which reduces myocardial inflammation and promotes tissue repair (Jia et al, 2016). Thus, Diabetes-induced cardiac inflammation plays a pivotal role in driving fibrosis and adverse remodelling. As such, suppression of inflammatory cell infiltration and/or inflammatory mediators may represent a viable therapeutic target for preventing or slowing development and progression of cardiomyopathy.
1.2.3.4.6 Inappropriate neurohumoral activation

Neurohormonal alteration is evident in the diabetic heart (van der Horst et al., 2010) with associated autonomic neuropathy as one of the complications leading to cardiac rhythm and vascular hemodynamic abnormalities. The prevalence of autonomic neuropathy in diabetic patients varies but may be as high as 60% (Fisher and Tahrani, 2017). It is characterised by increased β₁ adrenergic receptor expression and signalling due to sympathetic activation and upregulation of circulating catecholamines (C. Thomas, 2016), which promote cardiomyocyte hypertrophy, apoptosis, fibrosis and cardiac contractile dysfunction (Zhi et al, 2004). In contrast, reduced parasympathetic activation in diabetes is associated with low muscarinic receptor density but may also contribute to cardiac dysfunction (Olshansky et al., 2008). Autonomic dysfunction may manifest clinically as tachycardia and exercise intolerance which can induce silent myocardial infarction (Lee et al., 2019). In addition, increased vascular peripheral resistance with reduced elasticity may occur due to sympathetic nervous system dysregulation leading to reduced myocardial perfusion, ventricular arrhythmias and conduction abnormalities (Movahed, 2007). Moreover, mitral valve E/A ratio is known to be reduced in diabetic patients with autonomic neuropathy in association with diastolic dysfunction (Monteagudo et al., 2000).

1.2.3.5 Treatment strategies of diabetic cardiomyopathy

Despite lack of a specific therapy to treat or prevent diabetic cardiomyopathy, some strategies may be effective based on knowledge of the underlying pathogenesis. These includes lifestyle modification, improved metabolic control, and use of β-blockers and/or lipid-lowering agents to prevent or delay adverse cardiac remodelling and dysfunction. In addition, pharmacological agents may employ to combat the cellular and metabolic alterations underlying development and progression of diabetic cardiomyopathy, including fibrosis, inflammation and oxidative stress.

1.2.3.5.1 Lifestyle modification

Control of body weight and calorie intake combined with regular physical activity are associated with improved glycaemia, insulin sensitivity, post-receptor insulin signalling, and insulin-mediated glucose uptake (Chen et al., 2015a). In addition calorie restriction in type 2 diabetic patients and/or obese individuals ameliorates myocardial fatty acid metabolism and with improved left ventricular diastolic function.
(Hammer et al., 2008), and ejection fraction (Schrauwen-Hinderling et al., 2010). Physical activity also promotes beneficial effects on inflammation; specifically reduced pro-inflammatory cytokine secretion from immune and endothelial cells, together with promotion of anti-inflammatory cytokines such as adiponectin (Hopps et al, 2011). For example, the anti-inflammatory effects of physical activity have been demonstrated in Zucker Diabetic Fatty (ZDF) T2DM rats, in which regular aerobic exercise not only improved glycaemic control, but also reduced levels of proinflammatory cytokines such as C-reactive protein whilst increasing adiponectin level (Teixeira de Lemos et al., 2009).

1.2.3.5.2 Glycaemic control and antidiabetic medications
Multiple studies have revealed the key role of hyperglycaemia as a main driver of the pathogenesis of diabetic cardiomyopathy and vascular disease. Therefore, achievement of optimal blood glucose control positively impacts on cardiovascular morbidity and mortality. For example, multiple large-scale clinical trials, such as the UK Prospective Diabetes Study (UKPDS), have demonstrated increased risk of cardiovascular complications with poor glycaemic control (Holman et al., 2008) although other randomised control trials have reported apparently contradictory data, in showing no significant benefit of intensive glycaemic control on cardiovascular endpoints (Addison and Aguilar, 2011), which serve to highlight the complex relationship between glucose control and cardiovascular outcomes. Nonetheless, whilst the impact effect of glycaemic restriction on macrovascular complications may be debatable, it is more widely accepted to reduce microvascular complications, indicating that optimal glycaemic control may be beneficial for management and prevention of diabetic cardiomyopathy.

1.2.3.5.2.1 Sulfonylureas
Sulfonylureas such as glyburide, block ATP-sensitive K+ channels stimulating pancreatic insulin release and have been used to treat type 2 diabetes for over 50 years. However, they have been linked to an increase incidence of cardiovascular outcomes (Evans et al., 2006). Thereby are now only used in low risk patients.

1.2.3.5.2.2 Thiazolidinediones
Thiazolidinediones are insulin-sensitising agents which activate PPARγ (Quinn et al., 2008), thereby improving myocardial glucose utilisation and lipid profile. With regard to diabetes complications, they have been shown to also exert anti-inflammatory
effects associated with improved endothelial cell function and reduced cardiac hypertrophy and LV diastolic dysfunction (Ordu et al., 2010).

However, thiazolidinediones may also induce weight gain and increase renal water and sodium reabsorption, leading to systemic oedema and progression of heart failure symptoms (Chandra et al, 2017). Indeed, further to these effects, the thiazolidinedione derivative, rosiglitazone, was removed from the market because of its adverse cardiovascular events and potential risk of myocardial infarction (Loke et al, 2011). This has led to the Food and Drug Administration (FDA) now requiring preapproval or post-approval studies for all new antidiabetic medications in order to rule out cardiovascular risk (Hiatt et al, 2013).

### 1.2.3.5.2.3 Metformin

As an insulin-sensitising agent, metformin remains the drug of choice for glycaemic control in type 2 diabetes, especially for obese individuals despite increased risk of lactic acidosis (Defronzo et al., 2016). Metformin reduces hyperglycaemia by promoting GLUT4 translocation (Yang and Holman, 2006) via activation of 5-adenosine monophosphate-activated protein kinase (AMPK), thereby improves tissue and cardiomyocyte insulin sensitivity and glucose uptake. Notably, metformin is also known to exert anti-inflammatory and cardioprotective actions (Goodman et al., 2014).

### 1.2.3.5.2.4 Incretin based therapies

Glucagon like peptide-1 (GLP-1) is a peptide hormone secreted by intestinal L cells, which enhances insulin secretion in a glucose-dependent manner, thereby inducing satiety and controlling body weight (Cho et al, 2012). Further to these properties, it has been exploited as an incretin-based therapy for management of hyperglycaemia in type 2 diabetes patients. However, after secretion in to the circulation, endogenous GLP-1 is rapidly metabolised by dipeptidyl peptidase-4 (DPP-4) enzyme (Drucker and Nauck, 2006). Therefore, to overcome this short-half life, GLP-1 receptor agonists which are resistant to DPP-4, such as exenatide and liraglutide, were developed and are now being widely used (Prasad-Reddy and Isaacs, 2015).

Importantly, GLP-1 receptors are expressed on endothelial cells and cardiomyocytes and several studies have demonstrated cardioprotective effects of both GLP-1 analogues and DPP-4 inhibitors. For example, GLP-1 receptor activation and DPP-4 inhibition promote myocardial glucose uptake by enhancing insulin sensitivity, thus improving cardiac function (Grieve et al, 2009; Witteles et al., 2012), whilst GLP-1
receptor knockout mice show left ventricular hypertrophy by 5 months of age compared to wild type counterparts (Gros et al., 2003). Furthermore, chronic GLP-1 administration to hypertensive heart failure–prone rats is associated with reduced cardiomyocyte apoptosis and preservation of left ventricular mass index and function (Poornima et al., 2008a), whilst DPP-4 inhibition prevents cardiac hypertrophy and diastolic dysfunction in high fat/high fructose fed mice through suppression of oxidative stress (Bostick et al., 2014). GLP-1 also promotes anti-inflammatory effects on endothelial cells through suppression of NF-κB activation, and stimulation of nitric oxide (NO) induced vasodilation (Li et al., 2016), with similar beneficial effects observed with DPP-4 inhibition (Aroor et al., 2014). Taken together, these observations have led to the emergence of GLP-1 drugs as promising therapies for both metabolic and cardiac disease associated with type 2 diabetes.

1.2.3.5.2.5 Sodium-glucose cotransporter 2 inhibitors
Sodium-glucose cotransporter 2 inhibitors, such as empagliflozin, are a new class of antidiabetic agent (Neumiller, 2014), which increase renal glucose excretion through inhibition of sodium-glucose cotransporter 2 enzyme, thereby preventing glucose reabsorption (Kalra, 2014). As such, empagliflozin treatment is reported to reduce HbA1c, blood pressure, arterial stiffness, and visceral adiposity in T2DM patients (Inzucchi et al., 2015). Recently, the landmark Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients (EMPA-REG OUTCOME), reported reduced primary cardiovascular outcome and death compared to placebo, although incidence of genital infection was noted to be higher in empagliflozin treated group (Steiner, 2016).
1.2.3.5.3 Conventional therapies for diabetic cardiomyopathy

1.2.3.5.3.1 Lipid-lowering therapies-statins
Hyperlipidaemia is more harmful in diabetic patients than non-diabetics because of their higher propensity for atherosclerosis (Lee and Kim, 2017). Specifically, it has been recognised that the smaller size of low-density lipoprotein cholesterol (LDL-C) in diabetic individuals increases their vascular deposition, which is more atherogenic compared with similar plasma levels in non-diabetics. Indeed, statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors that reduce cholesterol biosynthesis, exert pleiotropic cardioprotective effects in diabetics even in the absence of established cardiovascular disease (Colagiuri and Best, 2002). In this regard, the Heart Protection study reported a reduction in cardiovascular events in diabetic patients without pre-existing coronary disease with daily simvastatin treatment over 5 years (Collins et al., 2003).

Although the key benefits of statins have been mainly attributed to their lipid lowering effects, some may be explained by other cellular mechanisms (Davignon, 2004). For example, statins increase the activity of eNOS, promoting vasodilation and reducing blood thrombogenicity by increasing nitric oxide bioavailability (Gelosa et al., 2007). In addition, they exert anti-inflammatory effects by reducing ICAM-1, VCAM-1, E-selectin, C-reactive protein and MCP-1 (Blanco-Colio et al., 2003). With specific regard to diabetic cardiomyopathy, fluvastatin is reported to attenuate cardiac dysfunction and fibrosis in an experimental rat model (Dai et al, 2011), whilst atorvastatin exerted similar cardioprotection which was attributed to its anti-inflammatory action (Van Linthout et al., 2007). Despite such data, there is a lack of clinical evidence supporting the benefits of lipid-lowering agents in individuals with established diabetic cardiomyopathy. Nonetheless, it is possible that hyperlipidaemia treatment to specifically control cardiac lipotoxicity may improve outcomes in diabetic cardiomyopathy patients.

1.2.3.5.3.2 RAAS-inhibition
Angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) are effective in controlling both micro and macrovascular diabetes complications (Cheng et al., 2014), with consequent suppression of cardiac fibrosis and hypertrophy by blocking the actions of angiotensin II (Kasznicki and Drzewoski, 2014).
Indeed, several independent clinical trials have confirmed the beneficial cardiac effects of ACEIs or ARBs. For example, the Heart Outcome Prevention Evaluation (HOPE) reported reduced rate of death, stroke and myocardial infarction in individuals with vascular disease or diabetes treated with ramipril (Wiklund, 2000). Indeed, ACEI and ARBs have been associated with several specific cardioprotective effects in diabetes, including amelioration of cardiac hypertrophy, fibrosis, and cardiomyocyte apoptosis (Scheen, 2004). In addition, ACEIs are reported to reduce blood pressure, enhance insulin sensitivity, improve glucose uptake (Shiuchi et al., 2002), and stimulate differentiation of adipocytes in T2DM (Sharma et al., 2002). Furthermore, ACEIs and ARBs inhibit the proinflammatory actions of angiotensin II; for example, decreasing TNF-α, IL-6 and IFN-γ levels in heart failure patients (Proudfoot et al., 2003). Taken together, these findings emphasise the clinical importance of targeting RAAS in diabetic patients, especially in those with cardiac hypertrophy and/or diastolic dysfunction.

1.2.3.5.3.3 β-blockers
β-adrenoreceptor blockers are a class of anti-hypertensive, anti-ischaemic, anti-arrhythmic agents, with anti-atherogenic properties. Their use has been recommended for many years for management of hypertension, myocardial infarction, and cardiac arrhythmia. (Čižmárková et al., 2019). In diabetic patients, clinical evidence favours the use of β-blockers in those with concomitant cardiovascular disease (López-Sendón et al., 2005), with the newer agents such as carvedilol particularly conferring beneficial effects, including improvement of both cardiac and systemic glucose metabolism, and reduction of myocardial oxygen demand (Cruickshank, 2002; Messerli and Grossman, 2004).

Therefore, at the very least β-blockers are safe for use in type 2 diabetic patients with heart failure (Wai et al., 2012). As such, use of β-blockers with favourable efficacy to improve metabolic profile in diabetic patients may be considered along with their standard application for hypertension, coronary artery disease and chronic heart failure unless specifically contraindicated.

1.3 The incretin axis and glucagon-like peptide-1 (GLP-1) biology
Clinicians are increasingly faced with the challenge of finding an antidiabetic agent that lowers blood glucose level while having beneficial effects on the cardiovascular system by counteracting the pathophysiological mechanisms and attenuating the
burden of risk factors which predispose to cardiovascular complications. In this regard, GLP-1 therapy shows great promise as a class of antidiabetic drugs that confer positive pleiotropic effects on the cardiovascular system.

1.3.1 Incretin physiology and effect
Glucagon like peptide-1 (GLP-1) is a 30 amino acid peptide, produced from the proglucagon gene located on the long arm of chromosome 2 (Baggio and Drucker, 2007a) and expressed in intestinal L-cells found in the distal ileum and colon from where GLP-1 is secreted (Ussher and Drucker, 2014). Whilst proglucagon is also expressed in α-cells of the pancreas and in the brain (Sinclair and Drucker, 2005), its cleavage is different between cell types due to tissue specific convertase (PC) enzymes (Tucker et al, 1996). In addition to GLP-1, L-cells synthesise other peptides, including GLP-2, glicentin and oxyntomodulin, via processing of convertase-1 (Holst and Holst, 2008). Initially, proglucagon generates GLP-1 (1-37) with subsequent formation of two circulating N-terminally truncated products, GLP-1(7-37) amide and GLP-1(7-36) amide (Cantini et al, 2016), the latter of which represents the majority of active GLP-1 in human circulation (Orskov et al., 1994).

The incretin phenomenon was established in the 1960s and describes the effect of an oral glucose load to promote insulin secretion at a significantly higher level versus isoglycaemic intravenous administration (Baggio and Drucker, 2007a). The incretin effect accounts for 50-70% of total insulin secretion, thereby playing a major role in maintaining glucose homeostasis. The incretin system consists of two gut hormones, glucose dependent insulinotropic polypeptide (GIP, originally referred to gastric inhibitory polypeptide) and glucagon like peptide-1 (Drucker, 2006), which are released from endocrine intestinal cells in response to food intake to enhance insulin release (Baggio and Drucker, 2007a), but interestingly, have opposing actions on glucagon secretion (Seino et al, 2010). In addition, GLP-1 exerts insulin-independent actions which further serve to control blood glucose by, for instance, delaying gastric emptying (Verspohl, 2009), decreasing appetite to induce satiety, and promoting weight loss (Hameed et al, 2009).

A particularly important feature of GLP-1 is that its metabolic actions on insulin and glucagon occurs in a glucose-dependent manner, this mitigating risk of hypoglycaemia. Consequently, the pleiotropic actions of GLP-1 on blood glucose control has gained significant attention as a novel treatment for type 2 diabetes mellitus over conventional therapies.
1.3.2 GLP-1 metabolism and elimination

GLP-1 can be eliminated from the circulation by 3 different mechanisms: renal clearance, hepatic clearance and circulatory degradation (Kieffer, 1999). Following its release into the circulation, GLP-1 is rapidly metabolised by DPP-4 through removal of an N-terminal dipeptide, generating GLP-1(9-36) and GLP-1(9-37) metabolites (Holst, 2007a), accounting for its short half-life of <2 minutes. Metabolically-inactive GLP-1(9-36) is reported to have 1000-fold lower affinity for the GLP-1 receptor, whilst lacking insulinotropic activity (Rolin et al., 2004).

1.3.3 GLP-1 receptor signalling

GLP-1 mediates its actions primarily via the GLP-1 receptor (GLP-1R) (Drucker and Nauck, 2006) which is expressed in pancreatic α- and β-cells, and other tissues/cells, including the heart, endothelium, vascular smooth muscle, macrophage, lung, kidney, and nervous system (Saraiva and Sposito, 2014). The highly ubiquitous nature of GLP-1R expression strongly indicates that it exerts important physiological effects other than glucose control, such as the increasingly apparent cardiovascular actions of GLP-1.

GLP-1R was originally identified in the 1990s from cloned rat and human cDNAs libraries derived from pancreatic islets as a G-protein coupled superfamily (Donnelly, 2012). Binding of GLP-1 to the GLP-1R activates the adenylyl cyclase pathway triggering intercellular cyclic AMP production (Burcelin, Gourdy and Dalle, 2014), thereby activating two cAMP binding proteins, protein kinase A (PKA) and cAMP-regulated guanine nucleotide exchange factor, also known as Epac2 (Holz, 2004). Although this is considered as conventional GLP-1 signalling, GLP-1R binding is also associated with activation of other kinase pathways, such as phosphatidylinositol-3 kinase (PI3K) (Drucker, 2006). GLP-1 has high affinity for the GLP-1R and is capable of binding at nanomolar concentrations, whilst other intestinal peptides, such as GIP, glucagon and VIP either poorly bind to the GLP-1R or not at all (Grieve, et al, 2009). Consequently, GLP-1 analogues have been designed to share structural homology with endogenous GLP-1 with comparable GLP-1R affinity. It should be noted that the GLP-1R is subjected to biased agonism, by which different GLP-1R analogues can induce diverse receptor-dependent signalling. (Fletcher et al., 2018).

1.3.4 Physiological actions of GLP-1

A wide range of physiological effects of GLP-1 on the pancreas and tissues have been reported, as summarised in Figure 1.7. As previously highlighted, the major
physiological action of GLP-1 is promotion of insulin secretion from pancreatic β-cells in response to increased glucose levels (Kieffer, 1999). It also exerts additional insulinotropic effects through promotion of insulin gene transcription and biosynthesis to replenish insulin stores (Wang et al., 1997), this increasing β-cell differentiation and proliferation and preventing apoptosis (Drucker, 2003). Indeed, gene-modified mice lacking GLP-1 protein display reduced insulin secretion and mild hyperglycaemia after intraperitoneal or oral glucose loading. GLP-1R/- mice exhibit decreased number of pancreatic islets with defective regeneration after partial pancreatectomy (De León et al., 2003).

Interestingly, in addition to its classical pancreatic actions, GLP-1 exerts numerous extra-pancreatic metabolic actions (Figure 1.7). For example, GLP-1 delays gastric emptying by vagal nerve inhibition (Holst, 2007b), inhibits hepatic glucose production, and promotes glucose uptake by peripheral tissues, such as liver and fat, whilst increasing sensitivity of skeletal muscle to insulin (Sinclair and Drucker, 2005). In addition, GLP-1 mediates central glucose control via stimulation of GLP-1Rs localised to hypothalamic nuclei which are important for satiety regulation (Drucker, 2006).

### 1.3.5 The incretin system in type 2 diabetes mellitus

Establishment of the pleiotropic actions of GLP-1 in blood glucose control has reinforced its potential for the treatment of patients with type 2 diabetes in which the incretin effect is defective compared to healthy individuals (Bagger et al., 2011)

![Figure 1-7: physiological actions of GLP-1 receptor activation. Adapted from (Grieve et al, 2009)](image)

Specifically, the insulinotropic effects of GIP are attenuated in T2DM patients, and even supraphysiological doses of GIP cannot further boost postprandial insulin release (Nauck et al., 1993). In contrast, although type 2 diabetic patients show
significant reduction in meal-stimulated GLP-1 release (Figure 1.8) (Toft-Nielsen et al., 2001), continuous infusion of GLP-1 normalises insulin responses to glucose load, suggesting GLP-1 activity is relatively preserved. Consequently, this has directed pharmaceutical efforts to harness GLP-1, rather than GIP signalling, as a treatment of diabetes. Importantly, the main obstacle preventing use of natural GLP-1 in the clinical setting is its short biological half-life (1.5–2 min). Thus, therapeutic targeting GLP-1’s insulinotrophic action is either achieved by direct stimulation of GLP-1 receptors using GLP-1 receptor agonists or mimetics which are resistant to DPP-4 activity (i.e. exenatide/Byetta®) or preservation of physiological levels of endogenous GLP-1 by inhibiting its metabolism using DPP4 inhibitors (sitagliptin/Januvia®) (Smits et al., 2016) (Figure 1.9).

GLP-1 and cardiovascular system

GLP-1 receptors are widely expressed in both the heart and vasculature of humans and rodents, with specific localisation in cardiomyocytes, endothelium and vascular smooth muscle (Ban et al., 2008). This highlights the now well-established role of GLP-1 in the cardiovascular system which necessitates detailed consideration of mechanisms by which GLP-1R agonists mediate their cardiovascular actions. As such, the use of incretin-based therapies for glycaemic control in T2DM patients has also been attributed to confer beneficial effects on CVD when compared to other antidiabetic medications. Indeed, numerous studies have reported the role of GLP-1 in modulation of heart rate and contractility, blood pressure and vascular tone (Ban et al., 2008) which may involve GLP-1R-dependent promotion of cardiac glucose uptake and NO production, suppression of triglyceride absorption and reduced inflammation (Okerson and Chilton, 2012).
For example, GLP-1 exerts positive inotropic and chronotropic effects via increasing cAMP and modulating cardiac Ca\(^{2+}\) current (Wang et al., 2013). Administration of native GLP-1 (7-36) amide to anesthetised rats produced dose dependent increases in heart rate and blood pressure (J. M. Barragan et al, 1994) which were not abolished by propranolol or reserpine, indicating these cardiovascular actions of GLP-1 occurred independently of the sympathetic nervous system (Baggio and Drucker, 2007b) Furthermore, administration of GLP-1R agonists is reported to increase systolic, diastolic and mean arterial blood pressure in rats, effects blocked by peripheral or central administration of exendin (9-39) as a GLP-1R antagonist, whilst gene-modified mice lacking GLP-1Rs displayed cardiac diastolic dysfunction associated with increased LV wall thickness (Gros et al., 2003). Such observations indicate that GLP-1R-dependent signalling plays a significant role in controlling cardiovascular structure and function. However, other studies have suggested that GLP-1 may exert its cardioprotective effects independently of the GLP-1R. For example, the GLP-1 metabolite, GLP-1(9-36), is reported to induce vasodilation effect via an NO/cGMP-associated mechanism that is independent of functional GLP-1Rs (Ban et al., 2008).
1.3.6.1 The effect of GLP-1 on risk factors for cardiovascular disease and diabetes

Hypertension, dyslipidaemia and obesity are established risk factors that contribute to cardiovascular disease in T2DM patients. Combined with hyperglycaemia, they greatly increase the risk of cardiovascular complications such as myocardial infarction, stroke and heart failure. It has been reported in both animal models and clinical trials that GLP-1-targeted therapies confer beneficial actions on such cardiovascular risk factors in diabetes (Grieve et al., 2009), which may be mediated by the established pleiotropic mechanisms of GLP-1 signalling (Figure 1.10). For example, in an experimental rat model, glucocorticoid-induced metabolic syndrome and associated hypertension was reduced by administration of the GLP-1 analogue, exenatide, for 7 days (Laugero et al., 2009). Exenatide administration to salt-sensitive db/db mice for 12 weeks also prevented angiotensin II-mediated increases in blood pressure. (Hirata et al., 2009).

Although chronic GLP-1 administration is reported to prevent hypertension development, acute GLP-1 exposure is associated with elevation of blood pressure and heart rate which may predispose to cardiovascular complications in diabetes. It has been found that short-term infusion of GLP-1 (7-36) and/or exendin-4 increased systolic/diastolic BP and heart rate of rats which were prevented by the GLP-1R antagonist exendin (9-39) pre-treatment (Barragán et al., 1996). This highlighting acute GLP-1R-mediated effects are insulin independent.
However, whilst the cardiovascular actions of GLP-1 in the experimental setting are well established, clinical trial data evaluating acute versus chronic GLP-1 administration on BP are variable. For example, GLP-1 infusion for 105 min in T2DM patients with coronary artery disease had no effect on heart rate and BP (Nyström et al., 2004a), while 48 hr GLP-1 administration to T2DM patients with ischaemic heart failure resulted in an increase in heart rate and BP (Halbirk et al., 2010). Although data from longer-term clinical trials are more consistent in demonstrating BP reduction with GLP-1 treatment, the underlying mechanism has not been clearly identified, with various pathways including direct arterial vasodilation, improvement of endothelial function, inhibition of RAAS, and GLP-1R-independent cGMP-mediated activation of nitric oxide having been proposed (Sun et al., 2015a). The Liraglutide Effect and Action in Diabetes (LEAD)-4 study which followed T2DM patients receiving liraglutide combined with metformin for 26 weeks reported a reduction in systolic BP compared with placebo (Zinman et al., 2009). Similarly, the LEAD-2 study which compared liraglutide with glimepiride therapy in T2DM patients reported a decrease in systolic BP (Nauck et al., 2013), whilst a meta-analysis of a 30 week trial comparing once-daily to twice-daily exenatide injection in T2DM patients established significant reduction in both systolic and diastolic blood pressure in both groups compared with baseline (Drucker et al., 2008).

It is possible to correlate BP reduction with chronic GLP-1 administration to its secondary metabolic changes, mainly weight loss, with BP reduction evident prior to significant weight loss. For example, a meta-analysis of 26-week liraglutide trials reported decreased BP after only 2 weeks, while significant weight loss was not apparent until 8 weeks (Gallwitz et al., 2010), suggesting that body weight reduction may be a secondary effect of GLP-1 treatment. Nevertheless, the weight-lowering effect of GLP-1 has beneficial actions on CV risk and diabetes.

For example, in experimental models, weight reduction is reported in heart failure/hypertensive rats treated with native GLP-1 in association with LV functional improvement (Poornima et al., 2008b). Similarly, native GLP-1 infusion in NHYA class III/IV heart failure patients for 5 weeks was correlated with 5 kg weight reduction (Sokos et al., 2006a), whilst the LEAD trials reported weight reduction following liraglutide treatment, particularly at 26 weeks (Wajcberg and Amarah, 2010). Importantly, GLP-1 agonists have several mechanisms to favourably ameliorate cardiovascular risk factors in diabetes especially when combined with other antidiabetic medications that may increase these risk factors.
Fat accumulation in T2DM is often associated with dyslipidaemia as a common proatherogenic cause of morbidity. Dyslipidaemia in type 2 diabetes is characterised by high triglycerides, low HDL-C, and changes in LDL-C structure towards small dense particles that favour atherosclerosis development (Buse et al., 2007). Combined with poor glycaemic control, dyslipidaemia is a major driver of atherogenesis (Sun et al., 2015b) Indeed, statins can reduce LDL-C, thereby decreasing incidence of cardiovascular events (Chilton et al., 2011), although substantial risk remains, perhaps due to other lipid abnormalities characteristic of type 2 diabetes (Farmer, 2008). In this regard, GLP-1R agonists have been established to improve lipid profiles in both experimental and clinical diabetes. For example, 4-week liraglutide treatment in rats with diet-induced obesity reduced circulating triglycerides and fat pad mass (Madsen et al., 2010), whilst exendin-4 administration for 40 days to T2DM mice improved dyslipidaemia and cardiac insulin resistance (Monji et al., 2013). Similarly, in clinical trials, twice daily exenatide (5 μg and 10 μg), extended-release exenatide, and 1.8mg liraglutide, are all reported to promote total cholesterol and triglyceride lowering (Sun, et al., 2015b). Furthermore, meta-analysis of the LEAD trials reported reduced triglycerides, total cholesterol, and LDL-C, with liraglutide compared with standard treatment (Sun et al., 2015b). Whilst LEAD-6 study highlighted superior reduction in triglycerides with liraglutide treatment compared with twice-daily exenatide (Plutzky et al., 2009). Similarly, potentiation of GLP-1 action by GLP-1R agonists or DPP-4 inhibitors is reported to decrease synthesis and release of chylomicron triglycerides, which promote atherosclerotic plaque formation, in both diabetic rodents (Hsieh et al., 2010), and humans (Matikainen et al., 2006), and to reduce hepatic fat and VLDL production (Parlevljet et al., 2012). Taken together, activation of GLP-1 signalling improves glycaemic control, body weight, blood pressure, and lipogenesis, thus highlighting multiple glycaemia-independent cardiovascular benefits of incretin-based therapies and further supporting their specific use for prevention and treatment of cardiovascular disease in diabetes.

1.3.6.2 GLP-1 and cardiovascular dysfunction
Numerous experimental studies have reported beneficial effects of GLP-1 treatment against acute myocardial ischaemia. For example, GLP-1 infusion in rats subjected to cardiac ischaemia-reperfusion reduced infarct size and improved post-ischaemic myocardial contractility (Bose et al., 2005). Similarly, GLP-1R agonist infusion in isolated rodent hearts prior to or concomitantly with ischaemia decreases infarct size
and promotes functional recovery (Drucker, 2016). Similar findings have been reported in the clinical setting with a randomised placebo-controlled trial indicating that exenatide administration for 3 days to patients with ST-segment elevated myocardial infarction reduced infarct size assessed by MRI and decreased circulating troponin I (Lønborg et al., 2012). In addition, liraglutide administration prior to and 7 days following percutaneous coronary intervention (PCI) improved left ventricular ejection fraction (LVEF) in both diabetic and nondiabetic subjects (Chen et al., 2015b). The proposed mechanism by which GLP-1R agonists confer cardioprotection effect against ischaemia may be related to their ability to increase myocardial glucose uptake rather than free fatty acid uptake, as glucose can efficiently generate adenosine triphosphate and carries more oxygen (Noyan-Ashraf et al., 2009).

In addition to the established infarct-limiting effects of GLP-1, our research group has reported that exendin-4 administration to mice subjected to permanent MI mice confers protection against chronic cardiac remodelling, independently of infarct size, which is particularly associated with marked attenuation of interstitial fibrosis, myocardial inflammation and decreased extracellular matrix gene expression (e.g. connective tissue growth factor, procollagen, fibronectin), with more modest reduction in cardiomyocyte hypertrophy/apoptosis (Robinson et al., 2015). It was therefore concluded that selective targeting of GLP-1 signalling to inflammatory and extracellular matrix pathways may promote effective post-MI cardioprotection by limiting potential detrimental mechanisms of cardiac changes in both diabetic and normoglycaemic subjects.

It seems likely that protection against adverse myocardial remodelling by GLP-1 at least partly involves modulation of endothelial function, which is compromised in diabetes and characterised by impaired coagulation and vasomotility, as well as increased release of proinflammatory mediators (Sheikh, 2013). As previously highlighted, endothelial dysfunction is an initiating cause of macrovascular and microvascular complications in diabetes, with several experimental and clinical studies reporting beneficial effects of GLP-1 based therapies. Indeed, GLP-1 agonists are reported to improve endothelium-dependent vasodilatation in type 2 diabetic patients (Tate et al., 2015), with the expected underlying mechanisms likely to involve regulation of key endothelial cell functions, such as promotion of nitric-oxide induced vasodilation, and inhibition of oxidative stress, smooth muscle cell proliferation and inflammation. Of these, the emerging anti-inflammatory actions may be most significant in mediating its cardioprotective actions. For example, liraglutide is
reported to ameliorate hyperglycaemia-mediated dysfunction in HUVECs via inhibition of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and plasminogen activator inhibitor-1 (PAI-1) (Liu et al., 2009). It has been suggested that liraglutide induces its anti-inflammatory actions via activation of eNOS and suppression of nuclear factor of NF-κB (Hattori et al., 2010), further supporting the supposition that key inflammatory modulatory effects of GLP-1 underlie its vasoprotective actions, although specific mechanisms remain unclear.

Similarly, whilst it is generally recognised that GLP-1 exerts direct vasorelaxant effects, several underlying mechanisms have been proposed. Some studies argue a role for GLP-1-dependent endothelial nitric oxide generation (Erdogdu et al., 2010), whilst others indicate the involvement of GLP-1R-independent β2-adrenoceptor activation (Green et al., 2008). Importantly, several published studies support a vascular role of GLP-1 in diabetes. Treatment with both GLP-1 and exendin-4 in streptozotocin-induced diabetes rats improves endothelial function and normalises vascular tone (Özyazgan et al., 2005), whilst liraglutide confers cardioprotective effects in apolipoprotein E-null mice via increasing eNOS expression and reducing endothelial ICAM expression (Gaspari et al., 2011). In humans, exendin-4 administration for 3-months in obese and pre-diabetic subjects is reported to improve microvascular endothelial function through reduction of inflammation and oxidative stress (Ceriello et al., 2011). Furthermore, type 2 diabetic patients with stable coronary artery disease show improved endothelial function, as assessed by brachial artery vasodilation, following GLP-1 administration, an effect not observed in non-diabetic subjects (Nyström et al., 2004b), supporting the theory that GLP-1-mediated vasoprotection may occur indirectly via insulin stimulation. In contrast, in ex vivo assessment of isolated aortic rings from Dahl salt-sensitive rats has indicated attenuation of endothelial dysfunction with GLP-1 administration, suggesting that vascular improvement may occur independently of its insulinotropic effect (Yu et al., 2003).

1.3.6.3 GLP-1 and cardiac remodelling
Whilst GLP-1R agonists have shown promising effects in animal models of heart failure, limited human data are available. The majority of experimental studies suggested that amelioration of insulin resistance by GLP-1 is the main mechanism underlying cardioprotection (Ingelsson et al., 2005). For example, exenatide infusion in a murine model of cardiomyopathy improved glucose tolerance and cardiac contractility (Kalla Vyas et al., 2011), whilst recombinant GLP-1 infusion for 48 h in a
canine model of pacing-induced dilated cardiomyopathy was associated with improvement of systolic and diastolic function (Nikolaidis et al., 2004). Furthermore, hypertensive, obese, insulin-resistant heart failure-prone rats treated with GLP-1 demonstrated increased myocardial glucose uptake, reduced cardiomyocyte apoptosis and preservation of cardiac function compared with vehicle-treated controls (Poornima et al., 2008a), suggesting that GLP-1 may improve cardiac function secondary to promotion of insulin-mediated glucose uptake. In the clinical setting, a small non-randomised study of patients with NYHA class III/IV heart failure reported that continuous GLP-1 infusion improved LV ejection fraction and increased myocardial oxygen consumption compared to HF patients on standard therapy alone (Sokos et al., 2006a). However, due to the limited number of clinical studies investigating effects of GLP-1 in heart failure, further research is needed to confirm these initial results, and those of more numerous experimental studies and to interrogate underlying mechanisms.

Although several experimental studies have indicated indirect cardioprotective actions of GLP-1, as previously highlighted, our research group has clearly shown that the GLP-1 analogue, exendin-4, exerts specific beneficial actions on cardiac inflammation and the extracellular matrix, independent of its established infarct-limiting or metabolic effects, which reduce adverse post-MI remodelling in mice subjected to chronic myocardial ischaemia (Robinson et al., 2015). Importantly, we extended these findings to the setting of experimental diabetes, in which we demonstrated similar attenuation of myocardial inflammation, fibrosis, and diastolic dysfunction in response to exendin-4, which occurred independently of its glucose-lowering and insulinotrophic effects. We reported specific attenuation of cardiac macrophage infiltration and pro-inflammatory cytokine secretion e.g. TNF, IL-6, and NF-κB, in association with reduced cardiac remodelling and improved function, strongly suggesting that specific targeting of inflammation-driven ECM remodelling may significantly benefit the diabetic heart.
1.4 Thesis aims and objectives

In conclusion, despite significant improvement in clinical management, cardiovascular complications remain the major cause of morbidity and mortality in type 2 diabetes (Angeli and Shannon, 2014). Whilst certain classes of antidiabetic medications reduce microvascular complications, they may increase cardiovascular risk (Nissen and Wolski, 2007). However, some drugs that target insulin resistance, such as metformin, to control associated metabolic disorders, and statins, and antihypertensive agents may ameliorate CVD development (Bistola et al., 2018). Nonetheless, effective treatment of cardiovascular disease remains a major challenge, largely due to the increasing prevalence associated with diabetes.

As such, gaining detailed understanding of the underlying mechanisms is critical in order to inform development of novel and more effective therapeutic strategies. In this regard, our research group has reported specific cardioprotective actions of the anti-diabetic peptide, GLP-1, which reduces myocardial inflammation, cardiac fibrosis, adverse remodelling, and particularly diastolic dysfunction, in response to both experimental MI and diabetes, independent of its infarct-limiting and glucose-lowering effects. Notably, complementary in vitro studies highlighted direct effects of exendin-4 on macrophages but not cardiac fibroblasts (which do not express the GLP-1R), indicating that it’s in vivo cardioprotective actions may be mediated by modulation of paracrine communication between infiltrating myocardial macrophages and resident fibroblasts, thereby benefitting ECM remodelling and diastolic function (Tate et al., 2016).

In this thesis, we therefore aimed to further explore mechanisms underlying the apparent cardioprotective effects of GLP-1 signalling in diabetes, focussing on the GLP-1 analogue, liraglutide, which is reported to reduce cardiovascular mortality in T2DM patients. Further to our previous data and the emerging literature highlighting modulation of inflammatory signalling by GLP-1, we chose to interrogate this specific aspect including effects on the vascular endothelium, as key pathways driving the development and progression of diabetic heart failure. In addition, we aimed to develop novel approaches for selective targeting of inflammatory cell GLP-1 signalling in order to maximise its evident therapeutic potential, whilst performing a small proof-of-concept clinical study to assess inflammation and cardiac function in T2DM patients receiving liraglutide versus standard therapy.
Therefore, the specific objectives of this thesis were:

1. To study the anti-inflammatory effects of the GLP-1 mimetic, liraglutide, on in vitro macrophage activation and paracrine communication with cardiac fibroblasts and endothelial cells in experimental diabetes.

2. To develop a macrophage-targeted GLP-1 peptide (modified liraglutide) with specific affinity for macrophages and assess to its biological activity in vitro and in vivo.

3. To investigate the clinical effects of liraglutide on circulating inflammatory cell profiles and cardiac function in patients with type 2 diabetes.
2 Materials and methods

2.1 Cell culture

Cell culture is the process in which cells are grown in a special aseptic artificial environment. Cells are subsequently maintained under carefully controlled conditions, which vary between cell types, but generally consist of a suitable medium that supplies the essential nutrients e.g. amino acids and regulates physio-chemical conditions e.g. HEPES, a pH buffer. In addition, cells are maintained in a special incubator which provides suitable temperature, gaseous environment and humidity to control their growth. Cell passage and seeding during experimentation are carried out in a biosafety cabinet (cell culture hood) as the most important equipment needed to prevent contamination.

Three mouse cell types were used in this thesis in order to assess intracellular signalling and paracrine communication between cells: immortalised bone marrow-derived macrophages (iBMDM), 3T3 fibroblasts, and immortalised cardiac endothelial cells.

2.1.1 Murine macrophages culture

2.1.1.1 RAW 264.7 macrophages

RAW 264.7 macrophages are a murine macrophages cell line obtained from the ascites of leukaemic virus-induced tumours in male mice; and were sourced from Sigma-Aldrich, UK. They were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 5.5mmol/L glucose with L-glutamine and pyruvate (ThermoFisher Scientific, UK) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were incubated in a T75 flask with 10ml media in an atmosphere of 5% CO₂ and at a temperature of 37 °C. Cells were passaged when they achieved approximately 80-90% confluency, which is usually achieved around 72 hours following previous subculture. They were split by aspirating the existing media, washing with 5ml PBS which was removed before 3ml DMEM culture media was added prior to dislodging the cells by scraping. Cells were moved from the T75 flask to a 15ml Falcon tube and centrifuged at 1300rpm for 5min. The supernatant was discarded, and the cell pellet was re-suspended in 10ml fresh media before being transferred to a new T75 flask, normally at a ratio of 1:10, depending on how rapidly they were required to achieve full confluence. Whilst RAW 264.7 cells were used at the beginning of this project, we later reverted to bone marrow macrophages as the
former demonstrated insufficient responses to allow assessment of our research questions.

2.1.1.2 Immortalised murine bone marrow macrophages

iBMDM were kindly donated by Dr. Adrien Kissenpfennig (Wellcome-Wolfson Institute for Experimental Medicine, Queen’s University Belfast). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 5.5mmol/L glucose with L-glutamine and pyruvate (ThermoFisher Scientific, UK) supplemented with 1% penicillin/streptomycin (ThermoFisher Scientific, UK) and 10% foetal bovine serum (ThermoFisher Scientific, UK), in a humidified atmosphere at 37°C and 5% CO₂. When they became confluent, cells were passaged by scraping and collected the cell suspension in a 15ml Falcon tube before centrifugation at 1500 rpm for 5 min. The supernatant was then discarded, and the cell pellet resuspended in fresh DMEM at the desired density. Cells were passaged at a 1:5 ratio for expansion in a T175 flask until they reached 90% confluency, when they were ready for sub-culture or seeding. Cells splitting was generally carried out twice per week (Figure 2.1).

Prior to experiments, cells were counted using an EVE automatic cell counter (NanoEnTek, Korea). This was achieved by mixing the cell suspension with Trypan blue at a ratio of 1:1 to discriminate dead cells and to determine cell viability before 10 µl was added to EVE chamber slide for counting.

For experiments, cells were seeded into 12-well plates at a concentration of 500,000 cells per well using 1ml culture media per well and were allowed to adhere overnight before adding the treatments.
2.1.1.3 Treatment of mouse macrophages

To mimic diabetes, RAW cells and/or iBMDM were cultured at high glucose concentration (25mmol/L) as our chosen experimental diabetic conditions. The basal concentration of D-glucose in standard DMEM culture media is 5.5mmol/L which is physiological in healthy subjects. High glucose media was prepared by dissolving 350mg D-glucose (Sigma-Aldrich, UK) in 10ml DMEM to generate a 10X stock solution (195mmol/L). After rigorous mixing to ensure complete glucose dissolving, the solution was sterilised by filtration with a 0.2μm syringe filter prior to dilution (1 in 10) in DMEM before adding to cells. High glucose treatment was performed at a final concentration of 25mmol/L D-glucose (5.5mmol/L in basal DMEM + 19.5mmol/L) in order to mimic uncontrolled clinical diabetes. Equimolar concentrations of L-glucose (25mmol/L i.e. 5.5mmol/L D-glucose + 19.5mmol/L L-glucose; Alfa Aesar, United States) were used as an osmotic control to allow for hyperosmolarity-induced cellular changes due to high D-glucose concentration. Importantly, D-glucose is a metabolisable glucose derivative, whilst L-glucose is a synthetic non-metabolisable glucose analogue, which cannot be utilised by living cells, so therefore does not impact upon glycaemia.

iBMDM were pre-incubated with the GLP-1 analogue, liraglutide (Synpeptide, China), with purity>90% (Lot No: JT63479) at a concentration of 10nmol/L, or exendin(9-39), as a GLP-1R antagonist (Synpeptide, China) with purity >90% (Lot No: JT63477) at a concentration of 100nmol/L. GLP-1 peptides were prepared in the standard culture media and added to the cells for 1h prior to the addition of 25mmol/L D-glucose (DG) in presence or absence of liraglutide (10nmol/L; DG+L), or liraglutide (10nmol/L) plus exendin(9-39) 100nmol/L for 48h. L-glucose was used as an osmotic control.
2.1.1.4 Harvesting of conditioned media

Macrophages secrete a variety of cytokines, chemokines and growth factors into their surrounding media under both basal conditions and in response to stimulation. Their secretome is known to alter the growth and function of other cell types through paracrine interaction both *in vitro* and *in vivo*. In order to interrogate the influence of GLP-1 peptides on iBMDM paracrine signalling, conditioned media from each treatment group was collected after 48h and stored at -80°C either for proteome profiler analysis or culture with cardiac fibroblasts and/or cardiac endothelial cells. When required, the media was thawed on ice and centrifuged at 4400rpm for 10min before being sterilised using a 0.22μm syringe filter prior to application.

2.1.2 Primary mouse cardiac fibroblasts

Primary mouse cardiac fibroblasts were kindly donated from Dr Kevin Edger at passage 1. They were cultured in DMEM (5.5 mM glucose) supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin. They were grown in 1% gelatin (Sigma-Aldrich, Poole, UK) pre-coated T25 flasks at 37°C with 5% CO₂. Once they become confluent (approximately 90%), media was removed and cells washed with PBS, detached using 0.25% trypsin-EDTA which was neutralised with excess media and centrifuged at 1200rpm for 5 min. The supernatant was discarded, and cell pellet resuspended in fresh cultured media for counting and then were seeded in 1% gelatin precoated 12 well plate at a density of 70,000 cell per well using 1ml culture media before they have been treated with RAW cells conditioned media to assess the paracrine signalling.

2.1.3 Murine 3T3 NIH fibroblasts

NIH-3T3 mouse fibroblasts were cultured in RPMI 1640 (ThermoFisher Scientific, UK) supplemented with 1% penicillin/streptomycin (ThermoFisher Scientific, UK) and 10% calf serum (Sigma-Aldrich, UK). Cells were cultured in 0.04% gelatin (Sigma-Aldrich, Poole, UK) pre-coated T75 flasks at 37°C with 5% CO₂ and split at a 1:10 ratio. For experiments, cells were used when they had reached a confluency of 80-90%. Trypsin-EDTA (0.05%; Gibco) was used to detach 3T3 cells and passage them in to new gelatin pre-coated T75 flasks either for sub-culture or experimentation (Figure 2.3). For experiments, cells were seeded into 12-well gelatin pre-coated plates at a concentration of 70,000 cells per well using 1ml culture media per well and maintained in the incubator until treatment with macrophage conditioned media to assess paracrine cells signalling.
2.1.4 Murine cardiac endothelial cells

Immortalised mouse cardiac endothelial cells (MCEC) were obtained from Cedarlane CELLutions Biosystems (Burlington, Canada). To characterise cardiac endothelial cells, they were stained with CD31 antibody before being imaged by confocal microscope using 40x magnification (Figure 2.4, A). Cells were maintained in DMEM with L-glutamine and pyruvate (ThermoFisher Scientific, UK) supplemented with 1% penicillin/streptomycin, 5% foetal bovine serum and 10mmol/L HEPES (ThermoFisher Scientific, UK) at 37 °C and 5% CO₂ under atmospheric oxygen concentrations. Cells were passaged every three days using Trypsin-EDTA (0.05%) to detach the cells prior to transfer to new T75 flasks (Figure 2.4, B). For experiments, cells were seeded into 12-well plates at a concentration of 70,000 cells per well using 1ml culture media per well and maintained in the incubator until they have been treated with macrophage conditioned media to assess paracrine cells signalling.

Figure 2-3: Representative images of NIH 3T3 fibroblasts using Leica light microscope 10X magnification.

Figure 2-4: Representative images of immortalised cardiac mouse endothelial cells (A) for characterisation, cells were stained with CD31 antibody (green) and DAPI (blue) for nuclear counterstaining then imaged by confocal microscope by 40x magnification (B) cells were imaged using Leica light microscope 10X magnification.
2.1.5 Macrophages conditioned media treatment

To assess paracrine interaction with other cell types, we employed a protocol which was developed in our laboratory in a previous study. Specifically, macrophage-conditioned media with or without exendin-4 was applied to cardiac fibroblasts to observe profibrotic changes (Tate et al, 2016), and we used a similar approach here.

After cardiac fibroblasts and endothelial cells were seeded for 24h, they were serum starved for 24h before addition of 1ml sterile macrophage-conditioned media collected from cells exposed to: L-glucose 25mmol/L, D-glucose 25mmol/L, D-glucose+ liraglutide 10nmol/L, or D-glucose +liraglutide+ exendin (9-39) 100nmol/L for 24 h. After incubation, conditioned media was removed, cells were washed with sterile PBS and were either fixed with 4% paraformaldehyde for staining or detached using 0.05% trypsin, collected in sterile Eppendorff tubes, and centrifuged at 1200 rpm for 5 min to obtain a cell pellet. Samples were stored at -80°C for real-time RT-PCR analysis to assess gene expression or western blotting for quantification of protein expression.

2.1.6 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs are comprised of monocytes, lymphocytes (T cells, B cells and NK cells), and dendritic cells. Density centrifugation medium (Ficoll-Paque) is the most commonly used method to isolate PBMCs using the underlying principle that cells are separated according to density gradient centrifugation.

Initially, 50ml Accuspin tubes (A2055-10EA, Sigma-Aldrich) were coated with Histopaque-1077(10771-500ML, Sigma-Aldrich) through addition of 15ml into the upper chamber prior to centrifugation at 2000rpm for 5min to allow the Histopaque-1077 to move in the chamber below the porous high density polyethylene barrier.

Once prepared, coated tubes were stored at 4°C until use (Figure 2.5-A)

40ml of patient blood was collected into a heparinised tube and diluted to double volume with Dulbecco's phosphate-buffered saline (DPBS) before 30 ml diluted blood was added slowly to the Accuspin coated tubes (Figure 2.5-B). Tubes were then centrifuged under the following conditions: 20°C, 480rcf, 20min, acceleration 5, brake 0. The PBMCs were identified as the white buffy layer (Figure 2.5-C) which was collected in to a sterile 50ml tube using sterile pasture pipette and topped up to 50ml with DPBS and centrifuged as follows: 4°C, 1200rpm, 5min, acceleration 9, brake 9.
The supernatant was then removed leaving behind the fragile pellet which was dispersed by gentle tapping, and the process repeated three times. After the last wash, the pellet was resuspended in 10ml RPMI, 1%FBS. For counting, cells were diluted 1:2 in an Eppendorff tube and 10µl were added to the haemocytometer before being incubated at 37°C for 5 min to allow monocytes to adhere.

Three cell types were visible - bright white lymphocytes, dark monocytes and dark small platelet dots, as indicated in Figure 2.6
2.2 Reverse-transcription polymerase chain reaction (RT-PCR)

Reverse-transcription polymerase chain reaction (RT-PCR) is a popular technique to study gene expression that has become highly advanced and has revolutionised molecular and biological research. Expression of genes is constantly changing under both physiological conditions and in response to stimuli, such as drugs, cytokines, or toxins. In this thesis, real-time RT-PCR was employed for accurate quantification of gene expression changes. Firstly, cellular RNA was isolated, followed by cDNA reverse transcription, and real-time RT-PCR which amplifies billions of copies of particular genes of interest for quantification relative to expression of a chosen housekeeping gene.

2.2.1 Extraction of nucleic acids

RNA was isolated from cells using a QIAGEN RNeasy kit according to the manufacturer’s specifications. In order to avoid any contaminating RNase activity, RNase-free water and RNase-free reaction tubes were used during the procedure. Briefly, for RNA extraction, cell pellet samples were thawed on ice before the addition of 350μl ice-cold lysis buffer containing 10μl/ml β-mercaptoethanol. Thereafter, an equal volume of 70% ethanol was added and mixed with the lysate which was transferred to the mini spin column and centrifuged for 15sec at 13000rpm, followed by two washes, repeat centrifugation steps and discarding of the flow-through after each wash. Finally, the mini spin column was transferred to a 1.5ml collection tube to which 30μl RNase-free water was added before centrifugation at 13000rpm for 1min to elute the RNA.

2.2.1.1 Determination of nucleic acid concentration

The concentration of nucleic acids was determined using the NanoDrop ND-1000 spectrophotometer with 1–2μl of sample (ThermoFisher Scientific, Loughborough, UK) by measuring light absorption at 260 nm. The Beer-Lambert equation was used to calculate sample concentrations. According to the Beer-Lambert (or Beer’s) Law, absorbance is proportional to concentration:

\[ A = \varepsilon bc \]

where \( A \) is absorbance.

\( \varepsilon \) is the molar absorptivity or extinction coefficient with units of L mol\(^{-1}\) cm\(^{-1}\).

\( b = \) path length, which is typically normalised to 1cm.

\( c = \) concentration of the compound in the solution.
In general, nucleic acid purity is determined by absorption at a wavelength of 230 and 280nm, respectively, with the A260/280 ratio used to calculate protein contamination of a nucleic acid sample. For pure RNA, A260/280 ratio should be somewhere around 1.8; a lower ratio indicates that the sample is contaminated with protein. The A260/230 ratio is a secondary measure of nucleic acid purity. Values are commonly in the range of 1.8-2.2. If the ratio is considerably lower, this may indicate the presence of co-purified contaminants, such as EDTA or phenol, which have absorbance close to 230nm. Together, this process was used to assess if the extracted RNA was of good enough quality for RT-PCR, with low ethanol and genomic DNA contamination, and to allow calculation of the volumes of sample required for reverse transcription, so that each sample of cDNA generated by RT would contain the same amount of cDNA. As such, this confirmed that any observed differences in RT-PCR gene expression were not due to differing amounts of DNA contained within each sample.

2.2.1.2 Estimation of RNA quality
It is important to determine the integrity of each RNA preparation, because degraded RNA does not perform well in RT-PCR analysis. RNA integrity analysis was performed by running the RNA on a 1% standard agarose gel electrophoresis. Briefly, 1g agarose was added to 100ml 1X Tris-acetate-EDTA (TAE) buffer and heated for 30 sec intervals. Once dissolved, Midori Green Nucleic Acid fluorescent dye (2μl) was added to allow detection of RNA bands through transillumination using the UVP imaging system (Upland, USA). Once cooled, the gel was poured into a cast tray containing a comb that was removed when the gel had solidified. A 1kb DNA ladder was loaded in to the first lane as an estimate of molecular size. RNA samples (1μl) were mixed with 6X orange dye (5μl) and loaded into the other lanes of the gel which was placed in a running tank filled with 1X TAE buffer and connected to electrical current at 100V for 1h. RNA integrity was established by observing the staining intensity of the major ribosomal RNA (rRNA) bands and any degradation products. A 28S:18S rRNA ratio of 2:1 is representative of good RNA quality. Partially degraded RNA lacks sharp rRNA bands or does not exhibit the typical 2:1 ratio associated with high quality RNA. Genomic DNA contamination in RNA samples can be visualised as it typically runs much more slowly through the gel matrix than uncontaminated RNA (Figure 2.7).
2.2.2 Conversion of RNA to cDNA

2.2.2.1 DNase Treatment

The presence of genomic DNA contamination in RNA samples is a frequent cause of false positive results in RT-PCR gene expression analysis. For these reasons, the inclusion of a DNase treatment step is often necessary. Therefore, before cDNA synthesis, all RNA samples were treated with a DNase kit (ThermoFisher Scientific, UK) to remove any traces of genomic DNA, in accordance with the manufacturer's instructions. To treat RNA with DNase, the following reaction was prepared in a 0.2ml PCR tube: 1µg RNA sample, 1µl 10x MgCl₂ reaction buffer, 1µl DNase buffer, and nuclease-free H₂O to a final volume of 10µl. The reaction tubes were incubated at 37°C for 30 min. After this time, 1µl EDTA was added to samples which were then heated to 65°C for 10min to inactivate the DNase prior to cooling to 4°C.

![Figure 2-7: RNA quality analysis using agarose gel electrophoresis](image)

(A) RNA generally shows two consecutive sharp and clear 28S and 18S bands in a 2:1 ratio (28S:18S), as indicated for samples 1 to 5 which shows that the RNA is completely intact. (B) DNA travels less as compared to RNA in same samples; in the case of DNA contamination, a DNA band is seen at the top of the gel along with RNA bands, as indicated in sample 3. Adapted from (Birtić & Kranner, 2006; Naderi et al., 2004)

2.2.2.2 cDNA synthesis.

Complementary DNA (cDNA) was synthesised using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems™ (Massachusetts, USA). Reverse transcriptase is an enzyme that translates RNA to DNA and creates one cDNA strand which is complementary to the RNA strand.

DNase treated RNA samples underwent cDNA synthesis using the reverse transcription protocol according to the manufacturer’s instructions: 2µl RT buffer, 0.8µl dNTPs and 2µl random primers from the kit and 4.2µl DEPC-treated water were added to each sample. After thorough mixing, 2µl was taken from each sample
as a negative control to confirm that there was no genomic DNA contamination so that the observed gene amplification was from reversed cDNA only. Reverse transcriptase (1µl) was then added to each sample in order to enhance cDNA production. All samples were incubated in a Veriti 96-well Thermal Cycler (Applied Biosystems) and run on the programmed detailed in Table 2.1.

Table 2-1: cDNA synthesis protocol

<table>
<thead>
<tr>
<th>Stage</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Stage 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature(°C)</td>
<td>25</td>
<td>37</td>
<td>85</td>
<td>4</td>
</tr>
<tr>
<td>Time (minutes)</td>
<td>10</td>
<td>120</td>
<td>5</td>
<td>∞</td>
</tr>
</tbody>
</table>

After synthesis, cDNA was further diluted 1:10 and stored at -20°C for subsequent analysis by real-time RT-PCR or conventional RT-PCR.

2.2.3 Primer design

Designing high-quality primers is one of the most crucial pre-experimental steps in RT-PCR. Specific primers for PCR should be designed carefully in order to eliminate the complications introduced with primer-dimers and non-specific product formation. Primers used in this thesis were designed using UCSC Genome Browser Blat, primer blast (NCBI) and primer3 online software, with consideration of criteria that were mentioned in table 2.2.

To avoid amplification of contaminated genomic DNA, primers should be designed so that they span an exon-exon junction; for example, one of the primers can be designed with one end complementary to the 3’ end of one exon and the other primer end complementary to the 5’ end of the next downstream exon.
Once primers had been designed, they were purchased from Integrated DNA Technologies (IDT, UK). They were reconstituted in DEPC water depending on the manufacturer's data sheet to achieve a 100µmol/L stock. For use, they were diluted in PCR tubes to a final concentration of 10µmol/L and stored at -20°C.

### Calculation of primers efficiency

It is important to measure the efficiency of PCR primers in order to calculate and analyse the results correctly. It is assumed that the efficiencies of PCR primers of genes of interest are comparable for the housekeeping gene during the calculation of delta-delta Ct values. Therefore, calculating primer efficiency is crucial to elucidate whether the high Ct values may be due to improper primer efficiency or low gene expression in the samples. Inappropriate PCR primer efficiencies within the experiment can significantly impact the results. Primer efficiency can be calculated by generation of a standard curve. To create a standard curve, it is recommended to perform serial dilutions of cDNA starting with the undiluted cDNA sample followed by 1:10 serial dilution with at least 4-points (Table 2.3)

#### Table 2-2: Factors to be considered for designing primers (Abd-Elsalam, 2003)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Optimal value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer length</td>
<td>18-22 base-pairs (BP)</td>
</tr>
<tr>
<td>Melting temperature (Tm)</td>
<td>59-60, primer pairs have Tm’s within 1°C of one another</td>
</tr>
<tr>
<td>% of GC content</td>
<td>45-55%</td>
</tr>
<tr>
<td>Self-complementarity</td>
<td>2</td>
</tr>
<tr>
<td>Self 3’ complementarity</td>
<td>2 or 3</td>
</tr>
</tbody>
</table>

Once primers had been designed, they were purchased from Integrated DNA Technologies (IDT, UK). They were reconstituted in DEPC water depending on the manufacturer’s data sheet to achieve a 100µmol/L stock. For use, they were diluted in PCR tubes to a final concentration of 10µmol/L and stored at -20°C.

### 2.2.4 Calculation of primers efficiency

It is important to measure the efficiency of PCR primers in order to calculate and analyse the results correctly. It is assumed that the efficiencies of PCR primers of genes of interest are comparable for the housekeeping gene during the calculation of delta-delta Ct values. Therefore, calculating primer efficiency is crucial to elucidate whether the high Ct values may be due to improper primer efficiency or low gene expression in the samples. Inappropriate PCR primer efficiencies within the experiment can significantly impact the results. Primer efficiency can be calculated by generation of a standard curve. To create a standard curve, it is recommended to perform serial dilutions of cDNA starting with the undiluted cDNA sample followed by 1:10 serial dilution with at least 4-points (Table 2.3)

#### Table 2-3: Example of serial dilution to get standard curve of 4 samples to check primer efficiency

<table>
<thead>
<tr>
<th>Tube</th>
<th>Sample</th>
<th>Volume of sample</th>
<th>Volume of PCR-grade water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cDNA (undiluted)</td>
<td>10µL</td>
<td>0µL</td>
</tr>
<tr>
<td>2</td>
<td>1:10</td>
<td>2µL (from tube 1)</td>
<td>18µL</td>
</tr>
<tr>
<td>3</td>
<td>1:100</td>
<td>2µL (from tube 2)</td>
<td>18µL</td>
</tr>
<tr>
<td>4</td>
<td>1:1000</td>
<td>2µL (from tube 3)</td>
<td>18µL</td>
</tr>
</tbody>
</table>
Samples were run in triplicate, in addition to a negative control sample containing PCR-grade water instead of the cDNA sample, to identify any contamination on the PCR plate. A Standard curve was calculated by plotting Ct values against the logarithm dilution factors. In the useable concentration range, there should be a linear relationship between Ct values and log dilution factor. The slope of the curve is the primer efficiency which should be close to 2.

**2.2.5 Real-time RT-PCR**

The process of real-time RT-PCR involves exponential amplification of billions of copies of the DNA sequence of interest or of a particular gene relative to a housekeeping gene through exposure of the double stranded DNA to different temperatures, thereby exploiting its stability changes. Real-time RT-PCR requires several components: a template strand of DNA, a polymerase enzyme, oligonucleotide primers to guide the amplification process, and nucleotides for polymerisation. Briefly, the process involves temperature cycling: a high temperature denatures the double stranded DNA into single stranded DNA, whilst a lower temperature allows primers to anneal, and strand extension by the polymerase enzyme requires a relatively higher temperature. The technique utilises SYBR Green fluorescent dye which binds to double stranded DNA and emits fluorescence in its bound form. In each real-time RT-PCR reaction cycle, the amount of DNA is amplified with a proportional increase in signal emitted from SYBR Green that is recorded as Ct (cycle threshold) (Figure 2.8).

Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold. Ct values are inversely proportional to the amount of target gene nucleic acid in the sample being amplified (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample).

In real-time RT-PCR, absolute quantification by SYBR Green requires primers of the gene of interest, and standard primers of a housekeeping gene, which is used as a reference to normalise transcription of the target gene.

RT-PCR reactions were carried out at a final volume of 2µl with the following mix: 1µl SYBR Green (LightCycler® 480 SYBR green 1 Master-Roche), 0.1µl of each forward and reverse primer (10μmol/L, Invitrogen/IDT), 0.6µl DEPC water and 0.2µl cDNA. A master mix was prepared for each target gene containing SYBR Green, forward and reverse primers and DEPC water depending on their proportions. The list of primers used in real-time RT-PCR experiments is detailed in Table 2.4.
All samples and SYBR Green master mix were loaded separately in to a 480 LightCycler 384 well plate using the Echo 525 Liquid Handler. After filling the plate and before loading in to the LightCycler 480II System, a sealed cover was used to seal the plate to prevent evaporation of samples, prior to centrifugation at 1200rpm for 2min to ensure sample sedimentation to the bottom of the wells. Samples were then run using the following programme in Table 2.5.

**Figure 2-8: Mechanism of SYBR® Green interaction with DNA.** SYBR® Green fluorescence (green star) increases when bound to double-stranded DNA compared with the unbound state (blue circle). The fluorescent signal increases proportionately as the RT-PCR amplification increases and is used to quantify the amount of DNA copies at the end of extension step in the RT-PCR reaction. Adapted from: (Botes et al 2013).

Denaturation:
Temperature is increased to separate DNA strands.

Annealing:
Temperature is decreased to allow annealing of primer to base pair to complementary DNA strand.

Extension:
Polymerase extends primer to form nascent DNA strand.
Table 2-4: List of primers used in RT-PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
<td>5’-GTGCTCCCGGGGCTGATT-3’</td>
</tr>
<tr>
<td>α-SMA:</td>
<td>5’-CTACGAAGTGCCTGACGGGC-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>CTGF</td>
<td>5’-GGTTCAAGACACAGAGGTGA-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>COL1α2</td>
<td>5’-TAGAAGAAAGCTGGCTGGCA-3’</td>
<td>5’-CGGCTGATGAGTCTTGGC-3’</td>
</tr>
<tr>
<td>MMP-2</td>
<td>5’-CCAGACAGGTCGAGCTGGAC-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>5’-CAGGTACAGAGGTGGCTGGT-3’</td>
<td>5’-CTCCCAATCCCTTGTGATC-3’</td>
</tr>
<tr>
<td>eNOS</td>
<td>5’-CTCTGAGACATCT ACT TCCC-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>Deoxatin</td>
<td>5’-CAGTCTTGCAGAATGGAAGGTTTGGAGA-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>Vimentin</td>
<td>5’-CAGCTTGCAGATCCCTGGAGA-3’</td>
<td>5’-AGTGGAGGATCAGCTTGAAC-3’</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>5’-TTTCCTCTGAGAAGCAGACACG-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>5’-GTGCAGTTTGAGAGGTGGTGTGGAGA-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>PAI</td>
<td>5’-GCCAGATTTATCATCAATGACTGGG-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>Sirtuin-1</td>
<td>5’-CAAGACCTCAAGCCATGTGTTGATA-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>ET-1</td>
<td>5’-CAAGGCCTGTTGCTGTTGCTTCT</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>Catalase</td>
<td>5’-CCTGTGCTGCTGCCTGCAACAG-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>IL-1beta</td>
<td>5’-TGCCACCTTCTTGAGATGTA-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’-GGAGATACATCCCTCCACAGAGG-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>IL-10</td>
<td>5’-TGCGAGGCTTGAAGGTTCTGAGG-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>IL-12</td>
<td>5’-GACATCATCAACACAGACCCAGGCC</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>CXCL-10</td>
<td>5’-GACGCTGGCTGCCGTCAACTG-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>NOX-2</td>
<td>5’-TGCGCAGTCTCAGAAGAGGTT-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
<tr>
<td>TGF-β</td>
<td>5’-TCAGGCTAGGTTGTACCCGCAC-3’</td>
<td>5’-GGTCCAGACACAGAGGTGA-3’</td>
</tr>
</tbody>
</table>

Gene abbreviations
- α-SMA: alpha-smooth muscle actin, CTGF: connective transforming growth factor; COL1α2: collagen1alpha2; MMP-2: matrix metalloproteinase-2; TIMP-2: tissue inhibitor of metalloproteinase-2; ICAM-1: intercellular adhesion molecule 1; VCAM-1: vascular cell adhesion protein 1; PAI: plasminogen activator inhibitor-1 ET-1: endothelin 1; eNOS: endothelial nitric oxide synthase; TGF-β: transforming growth factor beta; CXCL10: C-X-C motif chemokine 10.

Table 2-5: RT-PCR protocol used throughout this thesis

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Pre-incubation</th>
<th>Amplification</th>
<th>Melting curve</th>
<th>Cooling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>45</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Temperature</td>
<td>95°C</td>
<td>72°C</td>
<td>95°C</td>
<td>40°C</td>
</tr>
<tr>
<td>Time</td>
<td>10min</td>
<td>10sec</td>
<td>10sec</td>
<td>5sec</td>
</tr>
<tr>
<td></td>
<td>0.11sec</td>
<td>30sec</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2.6 Analysis of Quantitative RT-PCR

Analysis of real-time RT-PCR data was carried out using the Roche LightCycler 480 (1.5) software. For all relative quantification studies, the results were expressed as a
fold change \((2^{-\Delta\Delta C_t}}\) (Livak and Schmittgen, 2001) of gene amplification. Determination of threshold cycle (Ct) of all genes of interest was then normalised to the house keeping gene, \(\beta\)-actin or RPL-11. Initially, negative control samples and no reverse transcriptase samples were assessed to ensure that there was no amplification under basal conditions. Then, the mean Ct value for each gene from each sample triplicate was averaged and normalised to an endogenous housekeeping gene to obtain the \(\Delta\text{Ct}\) value, as follows:

\[
\Delta\text{Ct target gene} = \text{average Ct (control group)} - \text{Ct (sample)}
\]

\[
\Delta\text{Ct housekeeping gene} = \text{average Ct (control group)} - \text{Ct (sample)}
\]

\[
\Delta \Delta \text{Ct} = \Delta\text{Ct housekeeping gene} - \Delta\text{Ct target gene}
\]

Finally, the expression level of the target gene in a sample relative to the reference gene was calculated as fold change \(= 2^{\Delta \Delta \text{Ct}}\)

### 2.3 Western blotting

#### 2.3.1 Sample preparation

Also known as immunoblotting, western blotting is a common technique used to detect protein expression in cells or tissues. It depends on specific antibody binding to the protein of interest which are separated from one another according to their size by gel electrophoresis.

Firstly, media was aspirated from cells which were washed with PBS before being detached using 0.05% trypsin and centrifuged for 5min at 1500rpm. The resulting pellet was then washed with ice cold PBS and centrifuged at >11000rpm prior to resuspension by pipetting with ice-cold RIPA (Radio Immuno Precipitation Assay) buffer which was prepared by mixing the components as detailed in Table 2.6. Cell homogenates were vortexed every 10min for 30min at 4°C before centrifugation at >12,000rcf for 10min and collection of the protein-containing supernatant into clean Eppendorff tubes.
### Table 2-6: composition of RIPA buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl pH 8.0</td>
<td>500µl</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>88mg</td>
</tr>
<tr>
<td>sodium deoxycholate</td>
<td>50mg</td>
</tr>
<tr>
<td>Sodium dodecyl Sulphate (SDS) 10%</td>
<td>100µl</td>
</tr>
<tr>
<td>Igepal</td>
<td>100µl</td>
</tr>
<tr>
<td>Proteinase inhibitor</td>
<td>¼ tablet</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Up to 10ml</td>
</tr>
</tbody>
</table>

### 2.3.2 Protein quantification

The Pierce BCA Protein Assay was used to quantify protein concentration following the manufacturer's instructions (ThermoFisher Scientific, UK). The BCA Protein Assay relies on reduction of Cu²⁺ to Cu⁺ by protein in the sample in an alkaline medium (a temperature dependent reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu⁺) by bicinechonic acid. The level of reduced copper is proportional to the amount of protein in the sample, and detection of the cuprous cation, which causes a colour change from green to purple (Smith et al., 1985), with the water-soluble copper complex exhibiting a strong linear absorbance at 562nm. Samples (10µl) were loaded in to a 96-well plate in triplicate together with a broad working range (0-2000µg/µl) of bovine serum albumin standards, before 190µl BCA working reagent was added to each well using a multichannel pipette. The plate was then incubated at 37°C for 30min before absorbance was measured at 562nm using the FLUOstar Omega 37 microplate reader (BMG LABTECH, Germany). The concentration of the unknown samples was calculated from the standard curve generated using bovine serum albumin standards.

### 2.3.3 Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were separated based on their molecular weight using SDS-PAGE. Firstly, the gel casting equipment (Bio-Rad, Hemel Hempstead, UK) was thoroughly cleaned. Based on target protein molecular weight, a 12% acrylamide resolving gel for proteins ≤50KDa or 9% resolving gel for proteins >50KDa was then prepared, together with a 4% acrylamide stacking gel, as detailed in Table 2.7, and used to separate proteins. Once the resolving gel had been poured, tetramethylethlenediamime (TEMED) and ammonium persulfate (APS, 10%) were added as the last components to prevent early polymerisation. Once the resolving gel had set within ~45 min, the stacking gel was poured on top and either a 10- or
15-well 1.5mm comb was inserted into the top of the stacking gel, which was subsequently removed to form the wells for sample loading. Equivalent amounts of protein sample, typically 30µg, were prepared with 4x NuPAGE sample buffer (Invitrogen, NP0008, USA) containing freshly added 10x sample reducing agent (Invitrogen, NP0009, USA) and boiled for 5min at 95°C in a heat block, to ensure that proteins lost both their secondary and tertiary structure so that they could be separated on the basis of size. Once denatured, samples were carefully loaded into appropriate wells and a protein ladder (ThermoFisher Scientific, UK), as a molecular weight marker, was added to the first lane to determine the relative size of each protein.

The cast gels were then placed within a cassette and inserted into a Bio-Rad Mini ProTean Tetra cell running tank. The cassette and tank were filled with 1x running buffer (10x buffer: Tris 30g, Glycine 144g, SDS 10g). The running tank was then connected to a Bio-Rad PowerPac power supply and run at 150V for 1h or until the bands had reached the bottom of the resolving gel.

### Table 2-7: Composition of resolving and stacking gels for SDS-PAGE

<table>
<thead>
<tr>
<th>Component</th>
<th>12% Resolving gel</th>
<th>9% Resolving gel</th>
<th>4% Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O</td>
<td>8.5ml</td>
<td>11ml</td>
<td>6.1ml</td>
</tr>
<tr>
<td>1.5M Tris-HCl</td>
<td>6.25ml</td>
<td>6.25ml</td>
<td></td>
</tr>
<tr>
<td>pH 8.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5M Tris-HCL</td>
<td>-</td>
<td>-</td>
<td>2.5ml</td>
</tr>
<tr>
<td>pH 6.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrylamide</td>
<td>10ml</td>
<td>7.5ml</td>
<td>1.3ml</td>
</tr>
<tr>
<td>10% (W/V) SDS</td>
<td>250µl</td>
<td>250µl</td>
<td>100µl</td>
</tr>
<tr>
<td>10% (W/V) APS</td>
<td>130µl</td>
<td>130µl</td>
<td>50µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>15µl</td>
<td>15µl</td>
<td>10µl</td>
</tr>
</tbody>
</table>

### 2.3.4 Protein transfer

After completion of the running phase, the resolving gel was removed from the stacking gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon®, Sigma- Aldrich). Firstly, the membrane and filter papers were cut to the approximate size of the gel, before the membrane was activated with 100% methanol for 10-20sec, then equilibrated in 1x western transfer buffer (11.3g glycerine, 30g Tris base, 20% methanol) together with 4 pieces of Whatman filter paper. The resolving gel was then carefully placed on to the membrane within the cassette in the correct orientation and sandwiched between a double layer of filter paper and sponges to ensure proper transfer (Figure 2.9). The transfer cassette was
placed in a buffer tank Bio-Rad Mini-PROTEAN system with an ice pack filled with 1x transfer buffer and connected to a power pack at a voltage of 250mA for 90min (on ice).

![Transfer sandwich cassette](image)

**Figure 2.9:** Representative setup of transfer sandwich cassette for western blotting protein transfer phase Adapted from: (Liang et al, 2013).

### 2.3.5 Immunoblotting with specific antibodies

Firstly, the membrane was incubated in 5% non-fat milk in TBS containing 0.05% tween-20 (TBS-T) at room temperature for 1h to block any non-specific binding sites. The membrane was then incubated overnight at 4°C with the appropriate primary antibody diluted in 3% milk in TBS-T. The next day, six washes with TBS-T for 5min each were performed before addition of the species-specific HRP-conjugated secondary antibody, which was diluted 1:10000 in 3% milk in TBS-T, prior to incubation for 1h at room temperature.

After a further six washes in TBS-T, Immobilon western chemiluminescent HRP substrate (Millipore, UK) was used to detect the bands for visualisation. This was achieved by placing the membrane in the Syngene G:BOX Chemiluminescence Imaging System (Kodak, UK) and exposing the Immobilon western chemiluminescent mixture for band development. Visualised bands were then analysed using ImageJ software by measuring the area under the curve of band density with normalisation against the housekeeping control. A full list of antibodies used for western blotting in this thesis is presented in Table 2.8
Table 2-8: List of antibodies used for western blotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-smooth muscle actin</td>
<td>Mouse</td>
<td>1:1000</td>
<td>Dako (M0851)</td>
</tr>
<tr>
<td>DDR2</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Abcam, UK (ab126773)</td>
</tr>
<tr>
<td>Connexin-43</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Santa Cruz Biotechnology (sc-9059)</td>
</tr>
<tr>
<td>HPRT</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>Abcam, UK (ab10902)</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Cell signalling, USA</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Cell signalling, USA (82425)</td>
</tr>
<tr>
<td>β-actin</td>
<td>Mouse</td>
<td>1:5000</td>
<td>Cell signalling, USA (3700S)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Mouse</td>
<td>1:250</td>
<td>ThermoFisher Scientific, UK (MA5407)</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Abcam, UK (ab 174279)</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>Mouse</td>
<td>1:6000</td>
<td>Abcam, UK (ab52866)</td>
</tr>
<tr>
<td>Sirtuin-1</td>
<td>Mouse</td>
<td>1:1000</td>
<td>Abcam, UK (ab110304)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Cell Signalling, USA (2118)</td>
</tr>
<tr>
<td>GLP-1R</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Abcam, UK (ab39072)</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>ThermoFisher (61-7300)</td>
</tr>
<tr>
<td>Lamin A/C</td>
<td>Goat</td>
<td>1:1000</td>
<td>Santa Cruz Biotechnology (sc-6215)</td>
</tr>
</tbody>
</table>

**Secondary Antibody**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse IgG HRP-linked</td>
<td></td>
<td>1:10000</td>
<td>Cell signalling, USA (70765)</td>
</tr>
<tr>
<td>Anti-rabbit IgG HRP-linked</td>
<td></td>
<td>1:10000</td>
<td>Cell signalling, USA (70745)</td>
</tr>
<tr>
<td>Anti-goat IgG HRP-linked</td>
<td></td>
<td>1:10000</td>
<td>Abcam, UK (ab97110)</td>
</tr>
</tbody>
</table>

**Antibody abbreviations:**

DDR2: discoidin domain-containing receptor 2; NF-κB: nuclear factor kappa B; HPRT: hypoxanthine phosphoribosyltransferase; ICAM-1: intercellular adhesion molecule 1; VCAM-1: vascular cell adhesion protein 1; GLP-1R: glucagon like peptide-1 receptor; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; ZO-1: zonula occcludens-1; Phospho-eNOS: phosphorylated endothelial nitric oxide synthase; HRP: horseradish peroxidase.

### 2.4 Immunocytochemistry

Immunocytochemistry is a common laboratory technique used to visualise the expression and localisation of specific proteins in cells or tissues by employing a specific fluorophore primary antibody to facilitate visualisation of the protein(s) of interest under a fluorescence microscope or when the primary antibody binds to a conjugated fluorophore secondary antibody (Figure 2.10).
Generally, cells were grown on coverslips in 6-well plates for immunostaining. Media was aspirated, and cells were washed with PBS before they were fixed with 4% paraformaldehyde (PFA) for 20min at room temperature. After fixation, cells were washed twice with PBS and permeabilised for 10min at room temperature with 0.2% Triton X-100 in PBS. Cells were then blocked with 10% goat serum (Sigma-Aldrich, UK) in 0.1% Triton-PBS for 30min at room temperature and final washing with PBS followed by incubation overnight at 4°C with the relevant primary antibody (Table 2.9). The next day, cells were washed three times with PBS before being incubated with the appropriate secondary antibody in the dark at room temperature for 1h. After washing with PBS, cells were incubated with DAPI solution (1:1000; Sigma-Aldrich, UK) for 10min at room temperature for nuclear counterstaining, and again washed with PBS. Finally, the slide was mounted with Vectashield without DAPI (Vector Laboratories, USA) before the coverslip was added and the corners fixed in place with nail polish. Mounted slides were either stored at 4°C or directly imaged using a fluorescent microscope - either the DMi8 microscope (Leica Microsystems, Germany) or the Nikon confocal microscope (Nikon Instruments, USA). Antibodies that were used for immunocytochemistry in this thesis are detailed in Table 2.9.
Table 2-9: List of antibodies used for immunocytochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-smooth muscle actin</td>
<td>Mouse</td>
<td>1:100</td>
<td>Sigma-Aldrich, UK (C6198)</td>
</tr>
<tr>
<td>Connexin-40</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Santacruz Biotechnology (sc-365107)</td>
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<tr>
<td>CTGF</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Abcam, UK (ab6992)</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Abcam, UK (ab2314)</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Abcam, UK (ab 137321)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Mouse</td>
<td>1:200</td>
<td>ThermoFisher Scientific, UK(MA5407)</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Abcam, UK (ab 174279)</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Rabbit</td>
<td>1:200</td>
<td>ThermoFisher Scientific, UK (61-7300)</td>
</tr>
<tr>
<td>GLP-1R</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Abcam, UK (ab39072)</td>
</tr>
<tr>
<td>Sirtuin-1</td>
<td>Mouse</td>
<td>1:200</td>
<td>Abcam, UK (ab110304)</td>
</tr>
<tr>
<td>CD45</td>
<td>Rat</td>
<td>1:200</td>
<td>BD Pharmingen, UK (550566)</td>
</tr>
<tr>
<td>Goat anti-rabbit Alexa-fluor 594</td>
<td>1:500</td>
<td>ThermoFisher Scientific, UK(A32740)</td>
<td></td>
</tr>
</tbody>
</table>

Antibody abbreviations:
CTGF: connective transforming growth factor; ICAM-1: Intercellular Adhesion Molecule 1; VCAM-1: Vascular cell adhesion protein 1; ZO-1: Zonula occludens-1; GLP-1R: glucagon like peptide-1 receptor.

2.5 Flow cytometry

Flow cytometry is a widely employed method for identification of cell subpopulations based on their physical and/or fluorescent characteristics using prepared single cell or particle suspensions for flow cytometric analysis. Different immunofluorescent antibodies or dyes can be attached to the specific antigen(s) or protein(s) of interest. The cell suspension is aspirated into a flow system and passed in single file through a focused laser beam which is either absorbed or scattered when it strikes an individual cell. Absorbed light may be re-emitted as fluorescence if the cell is labelled with fluorophore antibodies or contains a naturally fluorescent substance. Light scatter is dependent on the cell size, shape and its internal structure. Fluorescent substances absorb light of an appropriate wavelength and re-emit light of a different wavelength. Various fluorophores may be used, including phycoerythrin (PE) and fluorescein isothiocyanate (FITC) as the most common fluorescent dyes in biomedical research. The resultant electrical pulses are digitised and displayed through a
computer system, and the obtained data provide quantitative information about every individual cell analysed, since large numbers of cells may be analysed in a short period of time.

Firstly, cells were prepared in suspension. The media was aspirated and cells were then washed with sterile PBS (Gibco™, ThermoFisher Scientific, UK), before being harvested by gentle scraping in the presence of flow cytometry staining buffer (FACs; FACS: PBS+0.5% FBS), rather than trypsin as a chemical detachment agent which may damage the cell surface proteins. After cells were collected, they were centrifuged, and the cell pellet was resuspended in FACS for cell counting to obtain an appropriate cell number; 1x10^5 cells /100µl FACs are commonly used for antibody staining. Cells were then incubated with the primary conjugated antibody prepared in FACS buffer at the recommended concentration for 15min in the dark at room temperature before they were washed 3 times by resuspension in a larger volume of FACs buffer (250-300µl) and centrifuged at 400g for 5min, with the supernatant discarded between washes. After the last wash, cells were suspended in 500µl FACS for being quantified on the flow cytometer and analysed using FlowJo software.

2.6 Flexstation (Ca^{2+}) assay

Calcium ions (Ca^{2+}) play a significant role in the control of key physiological processes, such as muscle contraction, cell metabolism, glandular secretion, cell survival and death. Therefore, changes in calcium may reflect cell pathological states or alteration of drug responses (Shaalan et al, 2017) which are most commonly measured using Fura-2 ratiometric fluorescent dye. Fura-2 exhibits an excitation spectrum including both bound Ca^{2+}, which is excited maximally at 340 nm, and free Ca^{2+}, which is excited maximally at 380 nm. Therefore, the concentration of free [Ca^{2+}] is proportional to the ratio of fluorescence at 340/380. To assess the hypothesis that inflammation and fibrosis may alter calcium signalling in 3T3 fibroblasts, we examined Ca^{2+} signalling using a FlexStation 3 Multi-Mode Microplate Reader.

To seed 3T3 FBs, a black plate (Molecular devices, USA) was coated with 0.04% gelatin before 10,000 cells were seeded per well in a volume of 100µl.

As described previously for the fibroblast and endothelial cell experiments, cells were serum-starved for 24h prior to incubation for 24h with macrophage-conditioned media collected from cells treated with L-glucose 25mmol/L, L-glucose + liraglutide 10nmol/L, D-glucose 25mmol/L, or D-glucose + liraglutide 10nmol/L.
After 24h, cells were washed with warmed 1x HBSS (ThermoFisher Scientific, UK). Initially, 50µl cell conditioned media was removed and 150µl HBSS was added, as a first wash to flush out the Phenol Red from the cells. A second wash was then performed by removing 150µl cell conditioned media and adding 150µl HBSS.

Finally, the previously added 150µl HBSS was removed resulting in a final volume of 50µl in each well. To prepare the FURA-2QBT dye (Molecular devices, USA), 10ml of 1X HBSS was added to the bottle with vigorous agitation for 1min before 50µl was added into each well and incubated at 37°C for 45min. In the source plate, ATP (Sigma-Aldrich, UK), as the test compound, was prepared at concentrations of 100µM and 10µM, with 100µl added per well by the machine using the loading tips. Then, the source plate, cell plate and loading tips were loaded in to the FlexStation 3 Multi-Mode Microplate Reader which had been pre-warmed to 37°C.

2.7 Barrier formation assay
Barrier formation was assessed in mouse cardiac endothelial cells using the xCELLigence Real Time Cell Analysis Instrument (ACEA Bioscience, USA). After they have been treated with macrophage-conditioned media for 24h, as previously described. Before seeding cells in the xCELLigence E-plate view 16 pet (ACEA Biosciences, USA), a background reading was performed using the xCELLigence system 1 sweep for 1min to ensure that there were no manufacturing errors in any wells of the plate. Cells then were counted and seeded at a density of 70,000 cells in 200µl per well and incubated for 20 min at the room temperature inside the cell culture hood before loading the plate in to the xCELLigence system. Barrier formation was recorded by assessing cell index (impedance) every 15 minutes until a plateau was reached indicating complete barrier formation.

2.8 Statistical analysis
Measurement from at least three biological replicates (n≥3) were statistically analysed using GraphPad Prism 7 software. Results are expressed as mean ± standard error of the mean (SEM). One-way or two- way ANOVA followed by post-hoc Bonferroni correction or unpaired Student’s t-test were used to compare data sets. After analysis, differences between groups were considered to be statistically significant when P<0.05.
3 In vitro examination of direct effects of liraglutide on macrophage activation and indirect effects on cardiac fibroblasts and endothelial cells in experimental diabetes

3.1 Introduction

T2DM has been classified as an inflammatory disease and many studies have established the role of inflammation as a central pathophysiologic process implicated in the development of diabetic cardiomyopathy, comprising cardiomyocyte hypertrophy, fibrosis, and endothelial dysfunction.

3.1.1 Signalling mechanisms underlying inflammation in the diabetic heart

Several cellular and molecular mechanisms have been implicated in accentuating the link between diabetes to myocardial inflammation. For example, hyperglycaemia and dyslipidaemia activate secretion of cytokines and chemokines e.g. IL-6 and monocyte chemoattractant protein (MCP-1) in cardiomyocytes and endothelial cells which worsen insulin resistance (Frieler and Mortensen, 2015), thereby promoting myocardial monocyte recruitment and myocardial inflammation. Activation of multiple signalling pathways, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which is a mediator of the inflammatory cascade (Figure 3.1) and renin-angiotensin-aldosterone (RAAS), have been implicated in the cardiac inflammatory response in diabetes (Reis et al., 2012). In inducible conditions, such as hyperglycaemia, NF-κB is translocated to the nucleus to trigger transcription of inflammatory cytokines and chemokines in endothelial cells, macrophages, and cardiomyocytes, including TNF-α which further activates NF-κB, thereby, intensifying NF-κB action in the diabetic heart. (Lorenzo et al., 2011). Indeed, activation of NF-κB modulates various biological responses, such as inflammation and cell death, thereby contributing to cardiac and vascular dysfunction (Hoesel and Schmid, 2013).

In addition, dysregulation of silent information regulator-1, known as sirtuin-1 (SIRT1) may promote development of myocardial and endothelial inflammation (Planavila et al., 2011). SIRT1 is a member of the class III histone deacetylase family, which requires NAD as a cofactor for its deacetylation effect (Sulaiman et al., 2010). It has been previously established that SIRT1 upregulation protects cardiomyocytes from oxidative stress, apoptosis and cellular aging (Hsu et al., 2010), whilst maintaining vascular homeostasis and regulating cardiac energy metabolism (Alcendor et al.,
The anti-inflammatory effect of SIRT1 is mediated through preventing activation of NF-κB and activator protein-1 (AP-1), thereby diminishing their transcriptional activation of multiple pro-inflammatory genes. However, hyperglycaemia reduces SIRT1 expression/activity which is reflected by increased NF-κB signalling (Sulaiman et al., 2010).

Figure 3-1: Molecular and cellular mechanisms underlying inflammation in diabetic cardiomyopathy and specific involvement of NF-κB and the renin-angiotensin-aldosterone system (RAAS).
Abbreviations: TNF-α, tumour necrosis factor-alpha, IL, interleukin; CRP, C-reactive-protein; PAI, plasminogen activator inhibitor; ICAM, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; TGF-β, transforming growth factor-beta. Adapted from (Reis et al., 2012)

3.1.2 The cell biology of diabetes-associated cardiac fibrosis

3.1.2.1 Macrophages and immune cells
Following these molecular events, leukocytes infiltration into the diabetic myocardium perpetuates the inflammatory process via secretion of a plethora of chemokines and cytokines which act synergistically to gradually promote progression of cardiovascular complications. In this regard, several studies have specifically demonstrated the involvement of pro-inflammatory mediators, such as IL-6 and C-reactive protein (CRP) (Pradhan et al., 2001). Whilst most leukocyte subtypes participate in diabetes-
associated inflammation, neutrophils, macrophages and T-lymphocytes, are thought to be particularly important, with macrophages playing a prominent role. Macrophages can be sub-classified into tissue-resident macrophages and infiltrating macrophages (Wynn et al., 2013). Tissue-resident macrophages maintain tissue homeostasis in response to changes in the external environment, whilst infiltrating macrophages present in response to tissue damage or inflammation (Davies and Taylor, 2015). In the early inflammatory phase, the presence of infiltrating macrophages and monocytes, which are predominant sources of inflammatory cytokines, has been established in myocardium in experimental type 1 and type 2 diabetes (Urbina and Singla, 2014; Tate et al., 2016). However, in the longer term pro-inflammatory cytokines expression may originate from the cardiac cells themselves (Turner et al., 2007). Both cardiomyocytes and cardiac fibroblasts appear to secrete pro-inflammatory cytokines and growth factors. Cardiomyocytes respond to inflammatory cytokines by undergoing hypertrophy or apoptosis, whilst fibroblasts differentiate to myofibroblasts with increased synthesis of collagen and fibronectin that ultimately lead to cardiac remodelling and heart failure (Jugdutt, 2003) (Figure 3.2). Importantly, these findings have been validated in patients with heart failure, with pro-inflammatory cytokines, TNF-α, IL-1, IL-6 and PAI, specifically recognised (Nian et al., 2004), confirming that inflammatory cytokines are implicated in diabetic cardiomyopathy development.

![Figure 3-2: The role of macrophage inflammatory mediators in cardiac fibrosis](image)

Thus, increased rate of heart failure in diabetic patients appears to involve hyperglycaemia-induced inflammation, mainly through activation of leukocytes,
thereby driving adverse remodelling and dysfunction. However, the precise underlying mechanisms remain incompletely understood.

3.1.2.2 Cardiac fibroblasts

The myocardium comprises distinct cell types including cardiomyocytes, cardiac fibroblasts, endothelial cells and smooth muscle cells, with cardiac fibroblasts (CFBs) considered as the largest cell type in the myocardium accounting for two-thirds of the cell population (Camelliti et al, 2005). CFBs are responsible for supporting heart structure by regulating mechanical, electrical and chemical signalling between cellular components (Krenning et al, 2010). They are functionally linked with cardiomyocytes via gap junction-mediated coupling proteins, mainly connexins (Cx40, Cx43, and Cx45), that maintain cardiac electrical conduction and synchronises contraction (Yue et al, 2011). CFBs secrete structural proteins to maintain ECM integrity, particularly collagens I and III and fibronectin (Kanekar et al., 1998), as a network which supports both cardiac structure and function (Krenning et al, 2010). The cardiac ECM also conveys intracellular signalling between cardiomyocytes, fibroblasts and blood vessels (Bowers et al, 2010). Importantly, CFBs secrete ECM regulatory proteases, such as matrix metalloproteinases (MMPs), which degrade ECM proteins, and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), which are important in homeostatic regulation of ECM composition, whilst imbalance may drive pathological cardiac remodelling (Visse and Nagase, 2003). Cardiac fibroblast functions are illustrated in Figure 3.3.

![Figure 3-3: Pleiotropic functions of cardiac fibroblasts](image)

Figure 3-3: Pleiotropic functions of cardiac fibroblasts. Cardiac fibroblasts communicate with cardiac cells through mechanical, chemical and electrical signals to maintain heart integrity. They have numerous functions as secretion of a number of bioactive molecules (such as growth factors and cytokines), ECM proteins, such as collagen, and matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) that regulate ECM integrity. Collectively, these pleiotropic functions impact upon cardiac structure and function in both health and disease. Adapted from: (Dostal et al, 2015)
In the infarcted and remodelling heart, fibroblasts respond to autocrine and paracrine signalling which activate migration and differentiation to myofibroblasts (Wang et al., 2003), which is accompanied by increased secretion of growth factors, cytokines, and metalloproteinases (MMPs), and deposition of ECM proteins, thereby promoting fibrosis (Figure 3.4). As they have morphological characteristics of both fibroblasts and smooth muscle cells, these cells were originally named as myofibroblasts by Gabbiani in 1972 (Gabbiani et al., 1972). They express smooth muscle cell markers, such as alpha-smooth muscle actin (αSMA) (Porter and Turner, 2009) but are more contractile and mobile because their microfilaments are connected to extracellular fibronectin through fibronexus as adhesion complexes (Yue et al., 2011), enabling ECM force generation which is maintained by collagen deposition (Gabbiani, 2003). Multiple studies have revealed that diabetes-induced hyperglycaemia promotes TGFβ transcription which subsequently increases TGFβ levels and downstream signalling, thereby driving CFB proliferation and differentiation (Dagher et al., 2017). As TGFβ specifically induces αSMA expression which induces collagen production, it has been suggested that αSMA detection may be used to identify collagen production after cardiac injury (Talman and Ruskoaho, 2016).

**Figure 3-4**: Fibroblast proliferation and differentiation to myofibroblasts. Differentiation of cardiac fibroblasts to myofibroblasts promotes ECM deposition, alteration of MMP and TIMP balance, thereby increasing expression of α-SMA and collagen deposition. α-SMA: α-smooth muscle actin, MMPs matrix metalloproteinases, TGF-β transforming growth factor β, TIMPs tissue inhibitors of matrix metalloproteinases. Adapted from (Talman and Ruskoaho, 2016)
Different in vivo diabetic models have demonstrated the effect of hyperglycaemia on cardiac fibrosis. For example, cardiac fibroblasts harvested from db/db type 2 diabetic mice demonstrated increased expression of collagen I, protease inhibitors and other profibrotic markers, such as TGFβ, in association with LV collagen accumulation and increased stiffness (Hutchinson et al., 2013). Moreover, fibroblasts isolated from hearts of obese diabetic Zucker rats exhibited high proliferative activity and elevated α-SMA expression, which is consistent with a myofibroblast phenotype (Fowlkes et al., 2013). Importantly, cardiac fibroblasts isolated from type 2 diabetic patients during coronary artery bypass surgery have also shown increased collagen I expression compared to non-diabetic donors (Sedgwick et al., 2014).

3.1.2.3 Endothelial cells

3.1.2.3.1 Physiology of the endothelium

The endothelium is the major determinant of vascular health. In this regard, Furchgott and Zawadzki (1980) reported the importance of endothelial-derived nitric oxide in regulating vascular tone (Furchgott and Zawadzki, 1980).

Indeed, the healthy endothelium produces a wide range of factors in response to chemical or physical signals to regulate cell adhesion, vascular inflammation and thromboresistance (Widlansky et al., 2003). Thus, endothelial cells are functionally active, promoting paracrine and endocrine signalling for maintenance of vascular homeostasis. Specifically, they regulate vascular cell growth, integrity, permeability,

Figure 3-5: Overview functions of endothelium. Adapted from : (Sena et al, 2013)
inflammation, and blood flow, in addition to maintaining blood fluidity (Widlansky et al, 2003) (Figure 3.5).

Impairment of the ability of endothelial cells to maintain vascular homeostasis is referred to as endothelial dysfunction, which has been linked to insulin resistance, type 2 diabetes, and obesity (Bakker et al., 2009). Indeed, the extent of endothelial dysfunction correlates to blood glucose level and individual insulin sensitivity (Cade, 2008) whilst endothelial dysfunction is established as a crucial mediator of associated atherosclerosis and cardiovascular disease. Key differences between healthy and dysfunctional endothelium are described in Table 3.1.

Table 3-1: Overview characteristics of endothelium function and dysfunction

<table>
<thead>
<tr>
<th>Healthy endothelium</th>
<th>Dysfunctional endothelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑ Vasodilatation (NO, PGI₂)</td>
<td>Impaired vasodilatation (↑ ET-1)</td>
</tr>
<tr>
<td>Anticoagulation, antithrombosis (NO, PGI₂, t-PA)</td>
<td>Coagulation, thrombosis (PAI-1, vWF)</td>
</tr>
<tr>
<td>↓ Oxidative stress ROS</td>
<td>↑ Increased oxidative stress ROS</td>
</tr>
<tr>
<td>Anti-inflammatory ↓ (CRP, MCP-1, TNF-α, IL-6)</td>
<td>Proinflammatory ↑ (CRP, MCP-1, TNF-α, IL-6)</td>
</tr>
<tr>
<td>↓ Adhesion molecules (ICAM-1, VCAM-1)</td>
<td>↑ Adhesion molecules (ICAM-1, VCAM-1)</td>
</tr>
<tr>
<td>↑ Repair and regrowth, ↓ damage</td>
<td>↓ Repair, ↑ damage</td>
</tr>
</tbody>
</table>

NO: nitric oxide; PGI₂: prostacyclin; ET-1: endothelin-1; t-PA: tissue plasminogen activator, C- PAI-1: plasminogen activator inhibitor; vWF, von Willebrand factor; ROS: reactive oxygen species; CRP: reactive protein; IL-6, interleukin-6; TNF-α, tumour necrosis factor alpha; MCP-1: Monocyte chemoattractant protein 1; ICAM: intercellular adhesion molecule-1; VCAM-1: soluble vascular cell adhesion molecule. Adapted from: (Sena et al, 2013)
3.1.2.3.2 Diabetes and endothelial dysfunction

The pathophysiology of vascular disease in diabetes largely involves endothelial cells, vascular smooth muscle cells and platelet dysfunction. Multiple studies have highlighted hyperglycaemia as a major factor in vascular disease development in diabetes, with both animal and clinical studies demonstrating that hyperglycaemia alters endothelial function and signalling, promoting low-grade inflammation, platelet activation, vasoconstriction, and vascular injury, which contribute to development and progression of associated microvascular and macrovascular complications (Creager et al., 2003).

The formation of advanced glycation end products (AGEs) impairs NO bioavailability and eNOS expression, whilst increasing expression of endothelin-1 (ET-1) in endothelial cells (Quehenberger et al., 2000). Additionally, hyperglycaemia promotes pro-inflammatory and pro-atherosclerotic responses in macrophages, endothelial cells and vascular smooth muscles cells (VSMC) (Brownlee, 2001), thereby promoting expression of adhesion molecules e.g. ICAM-1, and leukocyte-attracting cytokines in the vascular wall (Sena et al., 2013). In turn, high glucose can activate matrix metalloproteinases that induce vascular remodelling and plaque rupture (Sena et al., 2013). Therefore, determining the underlying mechanisms and sequence of events is important in order to inform development of new strategies for the prevention of cardiovascular complications associated with diabetes. Towards this ultimate aim, improved understanding and targeting of mechanisms underlying cardiac remodelling and endothelial dysfunction is pivotal. In this regard, inflammation is an emerging key driver of cardiac remodelling in diabetes which is preferentially altered by GLP-1 agonists. Specifically, our group has reported that exendin-4 preferentially modulates cardiac inflammation and extracellular matrix remodelling indirectly by targeting macrophages which are an essential component of cardiac remodelling in diabetes (Tate et al., 2016).

Although several large-scale trials have been performed to investigate cardiovascular actions of GLP-1 analogues in diabetes, the results have been variable. Whilst lixisenatide (ELIXA) and exendin-4 (EXSCEL) failed to show cardiovascular benefits (Schnell et al., 2016), liraglutide (LEADER) was reported reduced cardiovascular death and adverse events in patients with type 2 diabetes (Marso, et al., 2016b). Similarly, semaglutide (SUSTAIN) is associated with a reduction in cardiovascular events (Marso et al., 2016a), although cardiovascular outcome data are not yet available for albiglutide and dulaglutide (Prasad-Reddy and Isaacs, 2015). It is
possible that the reported differences between groups between GLP-1 analogues are related to differences in study design or population. However, this may not provide a clear explanation for the minimal clinical cardiovascular actions of lixisenatide and extended release exendin-4 versus the reported benefits of liraglutide and semaglutide. Further studies are therefore needed to elucidate the precise underlying mechanisms by which GLP-1 analogues modulate cardiovascular signalling in diabetes in both the experimental and clinical setting.

As a result, the specific aims of this chapter were to investigate the anti-inflammatory actions of GLP-1 analogues (focussing on liraglutide which has reported clinical cardiovascular benefit in diabetes) on macrophage activation and paracrine communication with both cardiac fibroblasts and endothelial cells as key aspects of adverse myocardial remodelling.

3.2 Materials and methods

Most of chapter generalised materials and methods are described in chapter 2. Culture details for RAW cells and iBMDMs, as a model of murine macrophages, as well as mouse fibroblasts and cardiac endothelial cells are described in detail in section 2.1.

3.2.1 Drugs used in the study

3.2.1.1 Exendin-4
Exendin-4 is a naturally-occurring GLP-1R agonist peptide of 39 amino acid which was obtained from the saliva of the Gila monster (Egan et al, 2002) and shares 53% amino acid homology with native GLP-1 (Gupta, 2013) (Figure 3.6). Unlike native GLP-1, exendin-4 is resistant to DPP-4 enzymatic degradation. Exenatide is a synthetic form of exendin-4, which was the first GLP-1R agonist approved by FDA in April 2005 for treatment of type 2 diabetes (Kazafeos, 2011) which is sold under the brand name Byetta® (Eli- Lilly) for twice-daily administration. Exendin-4 used in the study was provided by Synpeptide company, China, with purity >90% (Lot No: JT63478).

3.2.1.2 Liraglutide
Liraglutide is a long acting acylated GLP-1 analogue which shares 97% homology with native GLP-1 (Gupta, 2013). It was developed by Novo Nordisk and marketed under the brand name Victoza®, which was approved for use in the EU and Japan in 2009, and by the USFDA in 2010 (Mehta et al, 2017). Like exenatide, the prolonged
action of liraglutide is achieved by its resistance to DPP-4 enzymatic degradation. Specifically, addition of a palmitic fatty acid chain to lysine at position 26 (Figure 3.6) enables liraglutide to bind to albumin within the blood and subcutaneous tissue. As such, albumin controls the release of liraglutide at a constant rate, which slows its degradation and reduces renal elimination (Neumiller and Campbell, 2009).

Liraglutide used in the study was provided from Synpeptide company, China, with purity>90% (Lot No: JT63479).

3.2.1.3 Exendin (9-39)

In contrast to exenatide and liraglutide, exendin (9-39) is modulating peptide fragment of exendin-3 which was originally found in Heloderma lizards (Raufman, et al, 1991).

It acts as competitive GLP-1R antagonist which prevents binding of GLP-1 to its receptor, thereby inhibiting GLP-1/GLP-1R mediated signalling (Calabria et al., 2012). Exendin (9-39), as a GLP-1R antagonist, was provided by Synpeptide company, China, with purity >90% (Lot No: JT63477).

3.2.2 Expression of GLP-1 receptor (GLP-1R) in mouse macrophages and cardiac fibroblasts

RAW cell protein extract was prepared for western blotting as described in section 2.4 for detection of GLP-1R expression. Liver, kidney, brain and trachea were isolated from mice and homogenised in ice cold PBS. All were considered as GLP-1R positive controls except trachea which was used as a negative control. Protein concentrations

Figure 3-6 The molecular structure of GLP-1 peptides (A) human GLP-1; (B) exenatide; (C) liraglutide. The grey colour indicates the differences in the structure relative to human GLP-1. Adapted from (Knop et al, 2010).
of all samples were quantified by the BCA assay, and 25µg/ml loaded in to SDS-polyacrylamide gel as described in the western blotting section 2.4.3. A GLP-1R antibody (1:1000; Abcam, ab39072) was used to detect GLP-1R expression at a size of 53kDa, although most of the other GLP-1R antibodies tested were found to introduce non-specific bands. Immunocytochemistry staining for GLP-1R was performed as described in section 2.5 in RAW cells, iBMDMs and cardiac fibroblasts to assess GLP-1R expression. Cells were fixed with 4% PFA, permeabilised by 0.2% Triton X-100 in PBS, and blocked with 10% goat serum before being stained with GLP-1R antibody at a concentration (1:200). The recommended secondary antibodies were then added before DAPI stain was added to recognise cell nuclei.

### 3.2.3 Treatment of macrophages

Macrophage treatments are described in detail in section 2.1.1.3. Specifically, RAW cells were treated with either normal D-glucose (5.5mmol/L) as a basal control or high D-glucose (25mmol/L) to mimic experimental diabetes in the presence or absence of 1nmol/L exendin-4 or liraglutide. Cells were studied at different time points (24, 48, 72, and 96h) prior to assessment of mRNA expression of cytokines and chemokines and evaluate the anti-inflammatory effects of exendin-4 and liraglutide. After selecting iBMDM as a more relevant model of murine macrophages, initial experiments were performed to optimise the time point for macrophage treatment. iBMDM were treated with L-glucose (19.5mmol/L L-glucose+ 5.5mmol/L D-glucose) as an osmotic control, or D-glucose (25mmol/L) for 24h, 48h and 72h prior to collection of conditioned media. Based on our initial findings, subsequent experiments were performed on iBMDM treated with L-glucose (19.5mmol/L L-glucose+ 5.5mmol/L D-glucose) as an osmotic control, or D-glucose (25mmol/L) with/without 10nmol/L liraglutide in the presence or absence of 100nmol/L exendin (9-39) for 48h.

### 3.2.4 Harvesting of conditioned media

Macrophages secrete a variety of cytokines and chemokines into their surrounding media after they have been cultured under different conditions. This collected media was used for analysis of the secretome by proteome array to characterise cytokine and chemokine expression or for paracrine signalling studies using cardiac fibroblasts and/or endothelial cells. Specific details for media collection and sterilisation were previously described in section 2.1.1.2.
3.2.5 Paracrine signalling assessment between RAW cells and cardiac fibroblasts

An established laboratory protocol for macrophage-fibroblast paracrine interaction was employed whereby mouse primary macrophages were treated with normal glucose (5.5mmol/L) or high glucose (25mmol/L) with/without GLP-1 analogues for 72h before the conditioned media was collected to treat primary cardiac fibroblasts (Tate et al., 2016). As such, RAW cells and iBMDM were treated with normal glucose (NG, 5.5mmol/L) or high glucose (HG, 25mmol/L) in the presence or absence of 1nmol/L exendin-4 or liraglutide for 72h. Mouse primary cardiac fibroblasts were kindly provided by Dr Kevin Edgar (from our research group), when they were serum starved for 24h before treatment with RAW cell conditioned media for 24h. After exposure, cardiac fibroblasts were trypsinised and collected as a pellet for RNA extraction to assess profibrotic gene expression by real-time RT-PCR.

3.2.6 Assessment of liraglutide anti-inflammatory action through NF-κB nuclear activation

NF-κB activation plays a pivotal role in inflammation by inducing transcription of pro-inflammatory genes (Baldwin, 1996). To evaluate the effect of high glucose and liraglutide treatment on NF-κB p65 nuclear translocation in macrophages, iBMDM were seeded at a density of 5x10^5 per ml in 60mm culture plates overnight. The next day, cells were pre-treated with liraglutide at a concentration of 10nmol/L, or exendin(9-39) as a GLP-1 antagonist at a concentration of 100nmol/L. GLP-1 peptides were prepared in standard culture media and added to the cells for 1h prior the addition of 25mmol/L D-glucose (DG) in the presence or absence of liraglutide (10nmol/L; DG+L), or liraglutide with exendin (9-39) 100nmol/L; (DG+L+Ex9-39) for 24h. L-glucose (19.5mmol/L+5.5mmol/L D-glucose; LG) was used as an osmotic control.

After treatment, cells were washed with sterile PBS, scraped, and collected in a pellet for cytoplasmic and nuclear protein extraction using a Nuclear Extraction Kit (Abcam, ab219177) following the manufacturer’s instructions. Briefly, 500µL ice-cold Cytoplasmic Extraction Buffer was added per sample supplemented with 2.5µl of protease inhibitor and 2.5µl DTT. Each sample was then vortexed briefly before incubation on ice for 10 min, vortexing, and centrifugation for 3min at 1000g in a pre-cooled bench-top microcentrifuge. The supernatant from each sample was collected as the cytoplasmic lysate whilst the pellet was kept on ice for resuspension with 500µl ice-cold nuclear extraction buffer 1 supplemented with 2.5µl of protease inhibitor and
2.5µl DTT. All samples were kept on ice for 15 min with vortexing every 5 min prior to centrifugation for 3 min at 5000g in a benchtop microcentrifuge at 4°C and collection of supernatants as nuclear lysate. All protein lysates were stored at -80°C until use. Protein lysates were thawed and quantified using the BCA assay before 24µg cytoplasmic lysate and 14µg nuclear lysate were run on a 12% SDS-polyacrylamide gel and separated proteins transferred to a PVDF membrane, as described in section 2.4. After blocking with 5 % milk in TBS-Tween for 1h at room temperature, membranes were incubated overnight with rabbit NF-κB antibody (1:1000; Cell signalling, 8245). After washing, membranes were incubated with anti-rabbit HRP conjugated secondary antibody (1:10000; Cell signalling, 70745) for 1h at room temperature and the membrane developed with Western Lighting Plus ECL (Millipore).

3.2.7 Identification of molecular anti-inflammatory mechanism of liraglutide action in macrophages

iBMDMs were pre-treated with liraglutide at a concentration of 10nmol/L, together with IBMX, as a phosphodiesterase inhibitor 1mmol/L (I5879, Sigma) or MDL-12,330A, as a cAMP inhibitor 5µmol/L (M182, Sigma) for 1h prior to addition of 25mmol/L D-glucose (DG) in presence or absence of liraglutide (10nmol/L; DG+L), or liraglutide (10nmol/L) plus IBMX 1mmol/L (DG+L+IBMX), and/or liraglutide (10nmol/L) with MDL-12,330A hydrochloride 5µmol/L (DG+L+MDL) for 24h. L-glucose 25mmol/L was used as an osmotic control. Cytoplasmic and nuclear protein lysates were prepared as described previously for quantification of NF-κB expression by western blotting.

3.2.8 Proteome array

A Proteome Profiler™ antibody array (ARY006, R&D Systems, UK) was used to assess levels of secreted cytokines and chemokines in macrophage-conditioned media. Media from the same treatment group was pooled (n=5) from multiple repeated experiments. According to manufacturer’s instructions, membranes were blocked for 1h with Array buffer 6 before 1ml of each conditioned media was mixed with 500µL of Array buffer 4 and 15µL of reconstituted Mouse Cytokine Array Antibody Cocktail, and added to each membrane and incubated overnight at 4°C. Next day, the membranes were washed 3 times with 1X wash buffer before the diluted Streptavidin-HRP was added to each membrane and incubated for 30min at room temperature. Membranes were washed again before Chemi Reagent Mixture was added for 1min and the membrane developed using the Syngene G:BOX
Chemiluminescence Imaging System, after which pixel density of each spot was quantified using HLImage++ software (Western Vision Software, UT, USA).

### 3.2.9 Macrophage migration assay

To assess whether liraglutide may modulate peripheral macrophage infiltration as a potential mechanism underlying its reported cardioprotective actions, an in vitro migration assay was performed for iBMDM using the xCELLigence Real Time Cell Analysis system (ACEA Bioscience, USA) using the cell invasion and migration plate (CIM-Plate 16) after 48h treatment with LG 25mmol/L, DG 25mmol/L, DG+L 10nmol/L, or DG+L10nmol/L+Ex(9-39) 100nmol/L. The plate comprises an upper chamber in which the cells are placed and a lower chamber containing a chemoattractant, with the chambers separated by a polyethylene terephthalate (PET) microporous membrane through which the macrophages may migrate. 200µL cell media was added to each well of the CIM-Plate 16 (ACEA Biosciences, USA) for background reading using the xCELLigence system which performed 1 sweep for 1min to ensure that there were no errors in any well. 3ml of 48h macrophage-conditioned media was collected, that were rich in cytokines and chemokines were centrifuged at 3300rpm, sterilised with a 0.2µm filter and 160µL conditioned media was added to each well in the lower chamber to act as a chemoattractant. Cells were seeded in 200µL DMEM serum free media at a density of 2x10⁵ cells per well in the upper chamber in parallel with their conditioned media in the lower chamber. Cells then were incubated for 20 min at the room temperature inside the cell culture hood before the plate was loaded in the xCELLigence system. Macrophage migration was recorded by assessing cell index (impedance) every 5min until a plateau was reached.

### 3.2.10 Macrophage adhesion assay

After macrophages migrate, they adhere to other cells and secrete inflammatory cytokines and chemokines. Therefore, macrophage adhesion was assessed using the xCELLigence Real Time Cell Analysis system (ACEA Bioscience, USA) after 48h treatment. To mimic macrophage adhesion to cardiac tissue, the xCELLigence E-plate was coated with fibronectin (10µg/mL) as an extracellular matrix protein. Before adding cells, 200µL media was added to each well of the E-plate VIEW 16 PET (ACEA Biosciences, USA) for background reading using the xCELLigence system running 1 sweep for 1min to ensure that there were no errors in any well. The media was then aspirated and 1x10⁵ cells pre-treated with L-glucose 25mmol/L or D-glucose 25mmol/L ± liraglutide 10nmol/L or liraglutide with exendin(9-39) 100nmol/L for 48h
were seeded per well in 200µL of 48h macrophage-conditioned media collected from each treatment group. Cells were then incubated for 20 min at the room temperature inside the cell culture hood before the plate was loaded into the xCELLigence system and cell adhesion recorded by assessing cell index (impedance) every 15min until a plateau was reached.

3.2.11 Conditioned media experiments
After treating macrophages for 48h, conditioned media was harvested, as described in section 2.1.1.2, and incubated with either cardiac fibroblasts or cardiac endothelial cells after they were serum starved for 24h. Cells were then treated with macrophage-conditioned media for another 24h before they were processed for RT-PCR, western blotting, staining, Flexstation or barrier formation assay.

3.2.11.1 Evaluation of macrophage paracrine signalling effects on cardiac fibroblasts differentiation and ECM protein expression
To assess cardiac fibroblast differentiation after treatment with macrophage-conditioned media, mRNA expression of myofibroblast markers, alpha smooth muscle actin (α-SMA), connective tissue growth factor (CTGF), and ECM markers (MMP-2, MMP-9, TIMP-2) were quantified by real-time RT-PCR using the protocol described in section 2.2. Protein expression of myofibroblast markers, gap junction connexins, and Ca\(^{2+}\) channels were assessed by western blotting and immunostaining, as described in sections 2.3 and 2.4, respectively.

3.2.11.2 Evaluation of macrophage paracrine signalling effects on cardiac fibroblasts calcium handling
Alteration in Ca\(^{2+}\) signalling that may reflect impaired Ca\(^{2+}\) handling in fibroblasts in response to high glucose treatment and the influence of liraglutide was investigated using the Flexstation system, as described in section 2.6. Specifically, Ca\(^{2+}\) signalling in fibroblasts was assessed as response to glutamate 10-100µmol/L, and ATP 10-100µmol/L (Skiöldebrand et al., 2017). However, as initial experiments failed to produce a Flexstation response in cardiac fibroblasts to glutamate at any concentration, 100µmol/L ATP was used in subsequent experiments as the best mediator of Ca\(^{2+}\) release in cardiac fibroblasts. To assess the source of Ca\(^{2+}\), 3T3 fibroblasts were treated with normal glucose macrophage-conditioned media for 24h before pre-treatment for 1h with 20µmol/L cyclopiazonic acid (CPA), which inhibits sarcoplasmic reticulum Ca\(^{2+}\)ATPase thereby blocking Ca\(^{2+}\) release, followed by
addition of 10µmol/L ionomycin as a positive control and/or 100µmol/L ATP to assess fibroblasts Ca²⁺ release on the Flexstation system. For analysis of GLP-1-mediated macrophage paracrine signalling on cardiac fibroblast Ca²⁺ signalling, fibroblasts were incubated with L-glucose or D-glucose with/without liraglutide macrophages conditioned media for 24h prior the addition of 100µmol/L ATP for measurement of intracellular free Ca²⁺ concentration on the Flexstation system.

3.2.11.3 Assessment of macrophage paracrine signalling effects on endothelial cells activation

Endothelial cell dysfunction was assessed as mRNA expression of inflammatory adhesion molecules as ICAM-1 and VCAM-1, vasodilator-vasoconstrictor balance via mRNA expression of NO vs ET-1, in addition to the anti-inflammatory mediator, sirtuin-1, by real-time RT-PCR (section 2.2). As well, protein expression of inflammatory adhesion molecules was quantified by western blotting (section 2.3) and immunostaining (section 2.4), whilst endothelial cell barrier formation after treatment with macrophage-conditioned media was evaluated by the xCELLigence RTCA system (Section 2.7).

3.3 Results

3.3.1 GLP-1R expression was confirmed in mouse macrophages, but not 3T3 fibroblasts

Western blotting and immunofluorescence were performed to investigate whether the GLP-1R is expressed in RAW cells, iBMDMs and 3T3 fibroblasts. Representative images in Figure 3.7 demonstrate that iBMDMs and RAW cells expressed the GLP-1R, with reference to Chinese hamster lung fibroblasts (CHL) that were used as a positive control, whilst 3T3 fibroblasts did not express the GLP-1R. In addition, western blotting showed protein expression of GLP-1R in both RAW cells and 3T3 fibroblasts in comparison to brain, CHL, and BV2 mouse microglial cells, used as positive controls. We also used several other commercially-available GLP-1R antibodies which were found to be nonspecific, as previously reported (Huang et al., 2013).

3.3.2 Exendin-4 and liraglutide may modulate RAW cell cytokine and chemokine mRNA expression after exposure to high glucose

Type 2 diabetes is considered as a chronic pro-inflammatory disease that alters the balance between the M1 pro-inflammatory macrophage phenotype (which is activated) and the M2 anti-inflammatory macrophage phenotype.
(which is downregulated). As expected, there was variation in cytokine and chemokine expression between different time points after cells were treated with high glucose in the presence or absence of liraglutide and exendin-4. For example, mRNA expression of the pro-inflammatory cytokine, IL-1β, was significantly increased by high glucose at both 24h and 72h (P<0.001, Figure 3.8A) in comparison to normal glucose which was significantly decreased by liraglutide at both 24h (P<0.01, Figure 3.8A) and 72h (P<0.001, Figure 3.8A) and exendin-4 at 72h (P<0.001, Figure 3.8A). Moreover, mRNA expression of IL-6 was varied between time points. IL-6 is considered a proinflammatory and/or anti-inflammatory cytokines. Its expression was increased by high glucose treatment at 72h compared to normal glucose (P<0.05, Figure 3.8B), whilst liraglutide increased its expression when added to normal glucose media at 72h (P<0.01) and decreased its expression when added to high glucose media at 72h (P<0.05, Figure 3.8B). However, IL-12 mRNA expression was significantly higher in RAW cells after high glucose treatment versus normal glucose plus liraglutide at 24h (P<0.05, Figure 3.8C) and/or normal glucose plus exendin-4 at 72h (P<0.05, Figure 3.8C).

mRNA expression of the anti-inflammatory cytokine, IL-10, was significantly decreased in RAW cells after high glucose treatment at 96h (P<0.001, Figure 3.9A). However, liraglutide decreased its expression when added to normal glucose media at that time point (P<0.05), whilst exendin-4 also decreased IL-10 mRNA expression when added to normal glucose media at the same time point (P<0.001). Meanwhile, IL-10 mRNA expression was significantly higher in normal glucose with liraglutide in comparison to normal glucose with exendin-4 (P<0.05) at 72h, whilst the anti-inflammatory chemokine, TGFβ, was significantly increased in normal glucose compared to high glucose at 48h (P<0.05, Figure 3.9B). However, mRNA expression of TGFβ was significantly lowered by both exendin-4 (P<0.01) and liraglutide (P<0.05) under normal glucose conditions at 96h, whilst its expression was higher in normal glucose plus liraglutide versus normal glucose plus exendin-4 at 72 h (P<0.01). In addition, liraglutide but not exendin-4 increased TGFβ mRNA expression when added to high glucose treatment at 72h (P<0.001) compared to high glucose treatment. Furthermore, liraglutide addition to high glucose media increased TGFβ mRNA expression significantly when compared with exendin-4 addition to high glucose media at 72h (P<0.05, Figure 3.9B).
Figure 3-7: Representative images of GLP-1R expression in mouse macrophages and 3T3 fibroblasts. (A) Immunocytochemistry staining of GLP-1R (Green presented in RAW cells at 40X magnification) or (red presented in iBMDM, 3T3FB, CHL cells at 60X magnification) with DAPI nuclear counterstaining (blue) using confocal microscope. Chinese hamster lung (CHL) cells were used as a positive control. (B) Representative Western blot for GLP-1R expression in lane (1) brain, (2) CHL cells, (3) RAW cells, (4) BV2 macrophages, and (5) 3T3 fibroblasts (FB), with HPRT for normalisation.
3.3.3 High glucose-treated RAW cells elicit paracrine action on primary cardiac fibroblast differentiation which is modulated by liraglutide but not exendin-4

Our research group previously demonstrated that whilst exendin-4 did not exert direct effects on cardiac fibroblasts, it modulated macrophage secretion which indirectly altered fibroblast differentiation (Tate et al., 2016). We therefore harvested 72h macrophage-conditioned media which was used to treat primary mouse cardiac fibroblasts in order to investigate the influence of GLP-1 analogues on paracrine signalling in the setting of experimental diabetes. These experiments demonstrated that cardiac fibroblasts treated with conditioned media harvested from high glucose cultured RAW cells showed significantly increased mRNA gene expression of profibrotic procollagen 1α2 (Col1α2), whilst levels of procollagen 1α1 (Col1α1) and CTGF also tended to be increased (Figure 3.10B-D). Interestingly, whilst high glucose conditioned media alone had no effect on mRNA expression of α-SMA, this was significantly decreased by addition of liraglutide but not exendin-4 (Figure 3.10A), suggesting that liraglutide may modulate macrophage-mediated cardiac fibroblast differentiation in experimental diabetes.

3.3.4 High glucose-treated immortalised bone marrow-derived macrophages promote paracrine actions on 3T3 fibroblast differentiation

Initial experiments were performed to assess paracrine communication between immortalised bone marrow-derived macrophages (iBMDM) and 3T3 fibroblasts. Conditioned media collected from iBMDM exposed to high glucose for 24h and 48 h and incubated with 3T3 fibroblasts for a further 24h significantly increased mRNA expression of CTGF and procollagen 1α2 (Col1α2), but not α-SMA and MMP-9 (Figure 3.11A), whilst 48h macrophage-conditioned media resulted in upregulation of all profibrotic genes (Figure 3.11B) with mRNA expression of CTGF, α-SMA, and procollagen 1α2 (Col1α2) all reaching statistical significance. Therefore, 48h macrophages treatment duration was selected as the best time point for media collection for subsequent paracrine signalling studies with 3T3 fibroblasts. As 3T3 fibroblasts treated with 72h macrophage-conditioned media demonstrated detachment and increased cell death prior to collection for RT-PCR, these cells were discarded and this time point excluded for evaluation of 3T3 fibroblast profibrotic marker expression.
Figure 3-8: Effect of exendin-4, liraglutide on proinflammatory cytokines mRNA expression in RAW cell macrophages incubated in high glucose for 24h, 48h, 72h and 96h. RAW cells were cultured in normal/high glucose with/without exendin-4 and/or liraglutide (1nmol/L) for 4 different time points, prior to real-time RT-PCR analysis of mRNA expression of IL-1β, IL-6, and IL-12 (n=3). Each column represents mean ± SEM. Data were analysed by two-way ANOVA followed by Bonferroni’s post-hoc test. *P<0.05, **P<0.01, ***P<0.001. NG: normal glucose, NG+EX: normal glucose + exendin-4 1nmol/L, NG+L: normal glucose + liraglutide 1nmol/L. HG: high glucose, HG+EX: high glucose +exendin-4 1nmol/L, HG+L: high glucose + liraglutide 1nmol/L.
Figure 3-9: Effect of exendin-4, liraglutide on anti-inflammatory cytokines and chemokines mRNA expression in RAW cell macrophages incubated in high glucose for 24h, 48h, 72h and 96h. RAW cells were cultured in normal/high glucose with/without exendin-4 and/or liraglutide (1nmol/L) for 4 different time points, prior to real-time RT-PCR analysis of mRNA expression of IL-10, and TGF-β (n=3). Each column represents mean ± SEM. Data were analysed by two-way ANOVA followed by Bonferroni's post-hoc test. *P<0.05, **P<0.01, ***P<0.001. NG: normal glucose, NG+EX: normal glucose + exendin-4 1nmol/L, NG+L: normal glucose + liraglutide 1nmol/L. HG: high glucose, HG+EX: high glucose + exendin-4 1nmol/L, HG+L: high glucose + liraglutide 1nmol/L.
Figure 3-9: Effect of RAW cell conditioned media on differentiation of primary mouse cardiac fibroblasts. Media was harvested from RAW cells treated with normal/high glucose with/without 1nmol/L exendin-4 or liraglutide for 72h and incubated with murine cardiac fibroblasts for 24h prior to qPCR analysis of transcript levels of (A) α-SMA, (B) Procollagen Iα1, (C) Procollagen Iα2, and (D) CTGF (n=3). Each column represents mean ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni’s post-hoc test. *P<0.05 **P<0.01.
Figure 3-10: Effect of high glucose-treated macrophage-conditioned media on 3T3 fibroblast differentiation. To optimise the optimal time point for macrophage-conditioned media collection for 3T3 fibroblasts paracrine signalling studies, immortalised bone marrow-derived macrophages were treated with L-glucose (LG) or D-glucose (DG) for (A) 24h and (B) 48h, before conditioned media was harvested and incubated with 3T3 fibroblasts for 24h prior to mRNA expression analysis by qRT-PCR (n=3). Each column represents mean ± SEM. Data were analysed by unpaired Student's t-test. *P<0.05, **P<0.01. Cells were imaged using Leica light microscope at 10x magnification.
3.3.5 Liraglutide and exendin-4 treated iBMDM differentially affect paracrine regulation of 3T3 fibroblast differentiation in a dose-dependent manner

iBMDMs were chosen as our preferred macrophage model due to apparent variation in RAW cell responses with regard to gene expression changes and interaction with fibroblasts when they were treated with high glucose and GLP-1 analogues. Consistent with previous experiments, 3T3 fibroblasts incubated with 48h conditioned media from high glucose-treated iBMDM showed significantly increased mRNA expression of profibrotic genes α-SMA, MMP-2 and procollagen (Col1α1) (Figure 3.12A). Whilst addition of 10nmol/L liraglutide to high glucose-treated iBMDM significantly decreased paracrine-induced mRNA expression of all three profibrotic gens, with α-SMA, and procollagen (Col1α1) reaching statistical significance (Figure 3.12A), these effects were not seen with exendin-4 which oppositely increased mRNA expression of MMP-2 in 3T3 fibroblasts. In addition, increased protein expression of both α-SMA and DDR-2 observed in 3T3 fibroblasts after incubation with conditioned media from high glucose-treated iBMDM was clearly reduced with addition of liraglutide. Whilst liraglutide at 10nmol/L dose showed a clear modulation in DDR2, α-SMA expression. Therefore, in further studies to investigate the indirect cardioprotective effects of GLP-1R activation we decided to use liraglutide at a 10nmol/L dose as it clearly inhibited 3T3 fibroblast differentiation to myofibroblasts.

Selection of liraglutide as the focus of our research was also influenced by parallel nanoparticle drug modification experiments which found that liraglutide-modified PLGA nanoparticles did not alter drug-receptor interaction and activation (Figure 4.11, Figure 4.13) which was altered by addition of exendin-4 in PLGA nanoparticles (Figure 4.10).
Figure 3-11: Effect of liraglutide and exendin-4 on 3T3 fibroblast differentiation induced by conditioned media collected from high glucose-treated iBMDM. Conditioned media harvested from iBMDMs treated with L-glucose 25mmol/L or D-glucose 25mmol/L in presence/absence of exendin-4 (1, 10nmol/L) or liraglutide (1, 10nmol/L) for 48h was incubated with 3T3 cardiac fibroblasts for 24h prior to gene expression analysis by (A) qRT-PCR (n=3), and (B) western blotting with normalisation to HPRT. Each column shows mean ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni post-hoc test. *P<0.05, ***P<0.001. LG: L-glucose, DG: D-glucose, DG+Ex: D-glucose+ exendin-4, DG+L: D-glucose+ liraglutide.
3.3.6 Liraglutide attenuated hyperglycaemia induced inflammation in iBMDM by inhibiting nuclear NF-κB translocation

Hyperglycaemia initiates inflammatory signalling through over-activation of nuclear factor-kappa B (NF-κB) which in turn results in transcription of proinflammatory cytokines, chemokines, adhesion molecules, and inducible nitric oxide synthase, which promote development and progression of diabetic cardiomyopathy. Indeed, iBMDM exposed to 25mmol/L D-glucose for 24h demonstrated increased protein expression of nuclear(N- NF-KB) but not cytoplasmic NF-κB (C- NF-KB), which was prevented by co-treatment with liraglutide in a GLP-1R-dependent manner (Figure 3.13A), indicating that its reported anti-inflammatory effects may be mediated by amelioration of nuclear NF-κB activation. Further experiments performed to investigate the molecular mechanism underlying suppression of nuclear NF-κB (N- NF-KB) activation by liraglutide indicated that this effect was reversed by inhibition of cAMP using MDL12330A as adenylyl cyclase inhibitor, but not by inhibition of phosphodiesterase enzyme using IBMX that increased cAMP intracellular level (Figure 3.13B), suggest that liraglutide regulates macrophage inflammatory response via modulation of NF-κB nuclear activation through a cAMP-dependent pathway.

3.3.7 Liraglutide altered the expression of iBMDMs secreted inflammatory cytokines and chemokines in response to high glucose

When activated, macrophages secrete a range of inflammatory cytokines and chemokines which may altered structure and function of surrounding fibroblasts and endothelial cells, thereby influence tissue remodelling. To investigate the influence of GLP-1 signalling on macrophage paracrine communication, iBMDM were treated with high glucose in the presence or absence of liraglutide or exendin (9-39) for 48h prior to collection of conditioned media which was assessed for secreted cytokines and chemokines using a commercially-available Proteome Profiler™ array (R&D Systems) to reveal specific inflammatory mediators responsible for mediating the apparent inflammatory actions. Of the 40 protein spots present on the Proteome Profiler™ membranes, 10 were clearly detected after treatment with iBMDM conditioned media, as highlighted in Figure 3.14A, with quantification of expression shown in Figure 3.14B-C. These pooled data demonstrated that high glucose conditioned media appeared to induce upregulation of soluble intracellular adhesion molecule-1 (sICAM-1), and MIP-1α and MIP-1β as chemotactic chemokines which recruit inflammatory cells and increase production of inflammatory mediators (Driscoll, 1994), as well as IL-1 receptor antagonist (IL-1RA) which modulates the immune response, effects which were reversed by liraglutide and modulated by
exendin (9-39). Interestingly, whilst protein expression of MIP-2 and CXCL-10 were also increased by high glucose, they were further induced by addition of liraglutide. MCP-5, M-CSF, MCP-1, TNF-α, and CCL-5 were expressed at low levels which remained similar between groups.

Figure 3-12: Liraglutide prevents high glucose-induced inflammatory signalling in mouse immortalised bone marrow macrophages through modulation of nuclear NF-κB activation. (A) Mouse iBMDM were treated for 24h with (1) L-glucose (LG) 25mmol/L, (2) D-glucose (DG) 25mmol/L, or a combination of (3) D-glucose and liraglutide (DG+L) 10nmol/L, or (4) D-glucose and liraglutide (DG+L) 10nmol/L+exendin (9-39) 100nmol/L. (B) To identify the underlying molecular anti-inflammatory mechanism of liraglutide, mouse iBMDMs were treated for 24h with (1) L-glucose (LG) 25mmol/L, (2) D-glucose (DG) 25mmol/L, or a combination of (3) D-glucose and liraglutide (DG+L) 10nmol/L, (4) D-glucose plus liraglutide 10nmol/L and IBMX 1mmol/L, or (5) D-glucose plus liraglutide 10nmol/L, and MDL12330A 5μmol/L prior to detection of cytoplasmic NF-κB (C-NF-κB) and nuclear NF-κB (N-NF-κB) protein expression by western blot. Cytoplasmic NF-κB expression was normalised to GAPDH expression whist nuclear NF-κB expression was normalised to laminin A/C expression.
Figure 3-13: Cytokine/chemokine proteome array blots exposed to 48h iBMDM-conditioned media with quantification of protein expression using HLImage++ software (pooled from 5 preparations).

(A) Original proteome array blots; (B) quantification of protein expression using HLImage++ software. Black columns L-glucose control (LG); dark grey columns D-glucose (DG); intermediate grey columns high glucose +liraglutide (DG+L); light grey columns high glucose +liraglutide +exendin(9-39) (DG+L+Ex(9-39)).

(C) Protein detection presented as heat map (produced using Microsoft Excel); dark red colouring represents the highest and dark green colouring represents the lowest protein detection.

1:LG, 2:DG, 3:DG+L10nM, 4:DG+L+Ex(9-39) 100nM

95
3.3.8 Liraglutide modulates high glucose induced macrophage migration and adhesion

Infiltration and adhesion of macrophages to the myocardium, endothelium or adipose tissues initiates an inflammatory cascade which plays an important role in cardiovascular disease development in diabetes. To study the influence of GLP-1 signalling on these processes, iBMDM were treated with high glucose in the presence or absence of liraglutide and exendin (9-39) prior to assessment of cell index using the xCELLigence system. High glucose-treated macrophages showed increased migration towards macrophage-conditioned media which is rich in inflammatory cytokines and chemokines, such as Macrophage inflammatory protein-1 alpha (MIP-1α), which act as a chemoattractant and plays a role in macrophages recruitment, with comparison to L-glucose-treated control cells (Figure 3.15). This effect of high glucose was inhibited by liraglutide in a GLP-1R-dependent manner, as indicated by reversal with exendin (9-39) co-incubation. Similarly, high glucose treatment induced increased macrophage adhesion on to fibronectin coated E-plates as compared to L-glucose controls which was reduced by liraglutide and restored by exendin (9-39) (Figure 3.16).

Taken together, the results of this series of experiments suggest that liraglutide exerts its anti-inflammatory actions on high glucose-treated macrophages through modulation of NF-κB nuclear translocation leading to alteration of inflammatory mediator release and inhibition of macrophage migration and adhesion, which could at least partly explain its observed cardioprotective effects in diabetes.
Figure 3-14: High glucose-induced iBMDM migration is modulated by liraglutide. iBMDM were treated with L-glucose 25mmol/L (LG); D-glucose 25mmol/L (DG) in the presence or absence of liraglutide 10nmol/L (DG+L) with/without exendin (9-39) 100nmol/L (DG+L+EX(9-39)) for 48h. (A) Representative plot of macrophage migration assay based on impedance (cell index) measured by xCELLigence system over 20h (B). Mean cell index across all time-points as calculated by area under the curve for (C) migration and (D) adhesion. Each column represents mean ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni's post-hoc test * P<0.05 **P< 0.01, ***P< 0.001.
**Figure 3-15:** High glucose-induced iBMDM adhesion is modulated by liraglutide.

iBMDM were treated with L-glucose 25mmol/L (LG); D-glucose 25mmol/L (DG) in the presence or absence of liraglutide 10nmol/L (DG+L) with/without exendin(9-39) 100nmol/L (DG+L+EX(9-39)) for 48h. (A) Representative plot of macrophage adhesion to fibronectin CIM coated plate based on impedance (cell index) measured by xCELLigence system over 20h. (B) Mean cell index across all time-points as calculated by area under the curve for adhesion. Each column represents mean ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni’s post-hoc test * P<0.05 **P< 0.01, ***P< 0.001.
3.3.9 iBMDM paracrine signalling alters cell differentiation and function and is influenced by high glucose and liraglutide

3.3.9.1 High glucose-treated iBMDM influence cardiac fibroblast differentiation and ECM protein expression which is altered by liraglutide

Whilst it was previously demonstrated that cardiac fibroblasts do not express the GLP-1R, exendin-4 was shown to exert cardioprotective effects through indirect modulation of cardiac fibroblast differentiation (Tate et al., 2016). Consistent with this observation, conditioned media harvested from iBMDM after 48h treatment with D-glucose promoted fibroblast differentiation, as indicated by increased mRNA expression of pro-fibrotic genes, CTGF, α-SMA, and procollagen Iα2 (Figure 3.17 A-C), ECM remodelling markers, MMP-2 and MMP-9 (Figure 3.17E,F) and the intermediate filament protein, desmin (Figure 3.17H), changes which were attenuated by liraglutide and reversed by addition of exendin(9-39), indicating GLP-1R involvement. In contrast, conditioned media from high glucose-treated macrophages decreased mRNA expression of the intermediate filament vimentin (Figure 3.17G), and the TGF-β receptor antagonist, Smad7 (Figure 3.17I), although these changes were also altered by liraglutide and reversed with addition of exendin(9-39). Interestingly, the observed changes in profibrotic and ECM gene expression in response to high glucose and liraglutide occurred in the absence of any alterations in TIMP-2 expression.

Importantly, similar results were obtained in relation to protein expression with western blots and immunostaining indicating that conditioned media from high glucose-treated iBMDM increased expression of α-SMA, DDR-2, CTGF (Figure 3.18), whilst increased expression of fibronectin, an extracellular matrix (ECM) protein involved in cell–cell or cell-ECM adhesion, is reflective of fibroblast contraction capacity which determines fibrosis (Figure 3.19B). Notably, all these alterations in gene expression were attenuated by liraglutide. In addition, protein expression of the fibroblast marker, vimentin (Figure 3.19A), was decreased after incubation with high glucose macrophage-conditioned media, indicating that most of the fibroblasts were differentiated to myofibroblasts, compared to those treated with liraglutide, in which vimentin expression was preserved. Taken together, these data support the apparent beneficial effects of GLP-1R activation on fibroblast differentiation and ECM remodelling.
In addition, both western blotting and immunostaining indicated that expression of the gap junction protein, connexin-43 and connexin-40 which play crucial role in maintaining cardiac electrical conduction (Johnson and Camelliti, 2018), were reduced by high glucose iBMDM conditioned media but restored by liraglutide in a GLP-1R-dependent manner (Figure 3.20). This may be particularly significant as cardiac fibroblasts are thought to play an important role in modulating cardiac electrophysiological signals through communication with cardiomyocytes via gap junctions. These data therefore suggest that modulation of macrophage paracrine signalling by liraglutide may help to maintain fibroblast integrity, and thus support cardiac electrical conduction in diabetes, thereby preventing arrhythmias.
Figure 3-16: Effect of high glucose-treated iBMDM conditioned media on mRNA expression of activated 3T3 fibroblasts and the influence of GLP-1 signalling. iBMDM were treated with L-glucose (25mmol/L); D-glucose (25mmol/L) with/without 10nmol/L liraglutide in presence or absence of exendin (9-39) 100nmol/L for 48h before being harvested to treat 3T3 fibroblasts for 24h prior to qRT-PCR analysis of transcript levels of (A) CTGF, (B) α-SMA, (C) Col1α2, (D) TIMP-2, (E) MMP-2, (F) MMP-9, (G) vimentin, (H) desmin and (I) Smad7 (n=7). Each column represents mean ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni’s post-hoc test. *P<0.05, **P<0.01, ***P<0.001.
Figure 3-17: Effect of high glucose macrophage conditioned media on 3T3 fibroblast profibrotic protein expression and the influence of GLP-1 signalling. Representative profibrotic protein expression in 3T3 fibroblasts treated with iBMDM conditioned media; LG: L-glucose (25mmol/L), DG: D-glucose (25mmol/L), DG+L: D-glucose+liraglutide (10nmol/L), DG+L+Ex(9-39): D-glucose+liraglutide+exendin(9-39) 100nM for 24h. (A) α-SMA western blot expression was normalised to α-tubulin with protein quantification (n=4); and cells were stained for α-SMA expression (red) with DAPI nuclear counterstaining. (B) DDR-2 western blot expression was normalised to α-tubulin with protein quantification by image J software (n = 3); (C) Cells were stained for CTGF expression (green) with DAPI nuclear counterstaining (blue). Cells were imaged using Leica DMi8 microscope at 20x magnification.1: LG; 2: DG; 3: DG+L; 4: DG+L+Ex(9-39). Each column represents mean ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni's post-hoc test. *P < 0.05; **P < 0.01.
Figure 3-18: Representative immunocytochemistry images of 3T3 fibroblast activation markers after treatment with high glucose macrophage conditioned media with or without liraglutide and exendin (9-39)

3T3 fibroblasts were treated with macrophage conditioned media LG: L-glucose (25mmol/L), DG: D-glucose (25mmol/L), DG+L: D-glucose+liraglutide (10nmol/L), DG+L+Ex(9-39): D-glucose+liraglutide+exendin (9-39) 100nmol/L for 24h. Cells were stained for A: vimentin (red); B: fibronectin (green) expression with DAPI nuclear counterstaining (blue). Cells were imaged by Leica DMI8 microscope at 20x magnification,
Figure 3-19: Effect of high glucose macrophage conditioned media on 3T3 fibroblast connexin expression and the influence of GLP-1R signalling. A: Representative Western blot for connexin-43 in 3T3 fibroblasts treated with macrophage conditioned media; LG: L-glucose (25mmol/L), DG: D-glucose (25mmol/L), DG+L: D-glucose+liraglutide (10nmol/L), DG+L+Ex(9-39): D-glucose+liraglutide+exendin (9-39) 100nmol/L for 24h; blots were normalised to α-tubulin. B: Connexin-43 western blotting protein quantification by image J software (n=4). C: 3T3 fibroblasts stained for connexin-40 (red) to visualise gap junction expression with DAPI nuclear counterstaining (blue); and were imaged by Leica DMi8 microscope at 20x magnification. Each column represents mean ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni’s post-hoc test. **P < 0.01; ***P < 0.001.
3.3.9.2 Conditioned media from high glucose-treated iBMDM alters cardiac fibroblast calcium signalling and TRPV2 channel expression which is influenced by liraglutide

In addition to cardiac fibroblast differentiation, effects of macrophage paracrine signalling on fibroblast function was assessed by flexstation analysis to test the hypothesis that modulation of inflammatory mediators by liraglutide alters intracellular Ca\(^{2+}\) which may thereby influence ECM protein synthesis and degradation. Initial experiments used ionomycin as a positive control which creates pores in the cell membrane that are preferentially permeable to Ca\(^{2+}\), thereby increasing intracellular Ca\(^{2+}\), and Cyclopiazonic acid (CPA) as a specific inhibitor of the ER Ca\(^{2+}\)-ATPase. 3T3 fibroblasts were first treated with normal glucose macrophage-conditioned media with or without CPA pre-treatment to estimate and assess the source of Ca\(^{2+}\). As expected, ionomycin induced rapid Ca\(^{2+}\) influx through pores in the cell membrane and/or stores, as reflected by the peak phase, followed by cytoplasmic Ca\(^{2+}\) efflux, as reflected by the plateau phase, both of which were significantly decreased (but not completely abolished) by CPA pre-treatment (Figure 3.21A, C). Whilst these data confirm that ionomycin increases cytoplasmic free Ca\(^{2+}\) by increasing Ca\(^{2+}\) release from sarcoplasmic reticulum stores and increasing Ca\(^{2+}\) influx via cell membrane pores, these may not be the only sources of intracellular Ca\(^{2+}\) in 3T3 fibroblasts given that CPA caused only partial depletion of cytoplasmic Ca\(^{2+}\). Nonetheless, whilst ATP caused a similar pattern of activation, the Ca\(^{2+}\) signal was not completely abolished by CPA (Figure 3.21B, D) indicating that Ca\(^{2+}\) was solely derived from intracellular stores and membrane transport, at least in response to ATP. Moreover, fibroblasts treated with high glucose macrophage-conditioned media showed a significant increase in free Ca\(^{2+}\) (peak phase) in response to both ionomycin (10µmol/L) and ATP (100µmol/L) in comparison with L-glucose macrophage-conditioned media, which tended to be reduced by liraglutide (Figure 3.21E-H).

Whilst these initial studies suggest that liraglutide may influence paracrine modulation of fibroblast function by macrophages in experimental diabetes, further experiments should be executed in order to confirm these findings and to identify the main source of increased free intracellular Ca\(^{2+}\). Many G-protein-coupled receptors (GPCRs) are expressed in cardiac fibroblasts and are implicated in regulation of intracellular Ca\(^{2+}\) release, whilst P2Y receptors are linked to intracellular Ca\(^{2+}\) mobilisation and control of cardiac function; for example, stimulation of mitochondrial P2Y2 receptors is reported to increase fibroblast differentiation (Chen et al., 2012). Another suggestion is the involvement of activation of Ca\(^{2+}\) sensing receptors (CaR) in the mitochondria,
if they are expressed in fibroblasts (Zhang et al., 2014), which may promote increased intracellular Ca\(^{2+}\) and fibroblast differentiation through activation of MMPs proteases (Jourdan-LeSaux et al, 2010).

Notably, one of the proposed mechanisms underlying elevated free Ca\(^{2+}\) in high glucose-treated fibroblasts is increased expression of transient receptor potential cation channel subfamily V member 2 (TRPV2) channels. TRPV2 is a non-specific cation channel which is a part of the TRP channel family and allows the cell to communicate with its extracellular environment through the transfer of ions. TRP channels in general are non-voltage gated channels that are activated by a variety of stimuli including oxidative stress, thermal or mechanical stimuli or cell-metabolite accumulation. TRP channels are expressed in cardiac fibroblasts and are responsible for Ca\(^{2+}\) entry which plays an important role in fibroblast differentiation to myofibroblasts and secretion of ECM proteins (Yue et al, 2011). Indeed, in the present study, 3T3 fibroblasts treated with high glucose macrophage-conditioned media showed increased TRPV2 protein expression compared to L-glucose control by both western blot and immunocytochemistry (Figure 3.22), which was significantly reduced by liraglutide and partially restored by exendin (9-39). As such, it seems that TRPV2 expression may correlate with the observed excess Ca\(^{2+}\) influx in 3T3 fibroblasts as detected by Flexstation (Figure 3.21), so could be one of the targets of macrophage paracrine signalling in this setting.
Figure 3-20: Effect of high glucose macrophage-conditioned media and liraglutide on ionomycin and ATP-induced calcium release in 3T3 fibroblasts. Cells were treated with macrophage-conditioned media: N-glucose (NG), N-glucose+liraglutide (NG+L), L-glucose (LG), L-glucose+liraglutide (LG+L), D-glucose (DG), or D-glucose+liraglutide (DG+L) for 24h. To investigate the source of Ca\(^{2+}\), cells were pre-treated with cyclopiazonic acid (CPA) 20µmol/L (SERCA pump inhibitor) for 1h prior to exposure to ionomycin and/or ATP (A-D) or directly exposed to ionomycin and/or ATP in (E-H). Representative Flexstation traces of fura-2 fluorescence ratio measured in 3T3 cells following delivery of ionomycin 10µmol/L (A,E) and ATP 100µmol/L (B,F). Representative normalisation of fura-2 ratio peak and the plateau relative to baseline after ionomycin (C,G) and ATP (D,H). Each column represents mean ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni’s post-hoc test. *P < 0.05; **P < 0.01.
Figure 3-21: Effect of high glucose macrophage-conditioned media and liraglutide on TRPV2 channel expression in 3T3 fibroblasts. A: Representative Western blot for TRPV2 in 3T3 fibroblasts treated with macrophage-conditioned media; LG: L-glucose (25mmol/L), DG: D-glucose (25mmol/L), DG+L: D-glucose+liraglutide (10nmol/L), DG+L+Ex(9-39): D-glucose+liraglutide+exendin(9-39) 100nmol/L for 24h; blots were normalised to α-tubulin. B: TRPV2 western blotting protein quantification by image J software (n = 4). C: 3T3 fibroblasts stained for TRPV2 to visualise channel expression with DAPI nuclear counterstaining and were imaged by Leica DMi8 microscope at 20x magnification. Each column represents mean ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni's post-hoc test. **P < 0.01; ***P < 0.001.
3.3.9.3 Conditioned media from high glucose-treated iBMDM alters cardiac endothelial cell function which is influenced by liraglutide

In addition to our reported effects on fibroblast differentiation and function, conditioned media harvested from iBMDM after 48h treatment with L-glucose or D-glucose in the presence/absence of liraglutide (10nmol/L) and with/without exendin (9-39) altered endothelial cell function. Specifically, endothelial cell dysfunction due to high glucose conditioned media treatment was characterised by increased mRNA expression of adhesion molecules, ICAM-1 and VCAM-1 (Figure3.23A,B), and imbalance between eNOS (vasodilator) and ET-1 (vasoconstrictor) mRNA expression (Figure 3.23C,D), as well as increased mRNA expression of pro-thrombotic PAI (Figure 3.23E) and decreased the mRNA expression of anti-inflammatory sirtuin-1 (Figure 3.23F). Notably, all of these adverse gene expression changes were attenuated by liraglutide in a GLP-1R dependent manner with the exception of eNOS and sirtuin-1 which were increased after liraglutide treatment whilst PAI which remained increased after treatment with both liraglutide and exendin (9-39). Consistent with these mRNA expression data, western blotting and immunocytochemistry analysis indicated that protein levels of VCAM-1 and ICAM-1 (Figure 3.24) in cardiac endothelial cells were increased after incubation with high glucose macrophage-conditioned media and reduced by liraglutide, whilst sirtuin-1 protein expression showed opposite changes (Figure 3.25). In addition, although endothelial cell expression of the tight junction protein, ZO-1, was unaltered by treatment with high glucose macrophage-conditioned media, addition of liraglutide resulted in significantly increased levels which were reduced by exendin (9-39) (Figure 3.26). Complementary functional analysis indicated that conditioned media from high glucose-treated macrophages impaired endothelial cell barrier formation as assessed by xCELLigence (Figure 3.27), which was improved by liraglutide co-treatment and attenuated by exendin (9-39).

Taken together, these data suggest that GLP-1R activation in macrophages may protect the heart from high glucose-induced adverse remodelling through modulation of endothelial cell function and fibroblast differentiation in a NF-κB dependent manner. As such, these findings support our suggestion that liraglutide confers anti-inflammatory effects which mediate crucial cardioprotective benefits in diabetes.
Figure 3-22: Effect of high glucose and liraglutide iBMDM conditioned media on cardiac endothelial cell mRNA expression. iBMDMs were treated with L-glucose (25nmol/L); D-glucose (25nmol/L) with/without 10nmol/L liraglutide in the presence or absence of exendin(9-39) 100nmol/L for 48h before being harvested to treat murine cardiac endothelial cells for 24h prior to qRT-PCR analysis of transcript levels of (A) ICAM-1, (B) VCAM-1 (C) ET-1, (D) eNOS, (E) PAI-1, (F) Sirtuin-1 (n=7). Each column represents mean ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni's post-hoc test. *P<0.05, **P<0.01, ***P<0.001.
Figure 3-23: Effect of high glucose macrophage-conditioned media on murine cardiac endothelial cells adhesion molecule protein expression and the influence of liraglutide. Representative adhesion molecule protein expression in cardiac endothelial cells treated with macrophage-conditioned media; LG: L-glucose (25mmol/L), DG: D-glucose (25mmol/L), DG+L: D-glucose+liraglutide (10nmol/L), DG+L+Ex(9-39): D-glucose+liraglutide+exendin(9-39) 100nmol/L for 24h. (A) VCAM-1 western blot expression was normalised to α-tubulin with protein quantification (n=4); and cells were stained for VCAM-1 expression (red) with DAPI nuclear counterstaining (blue). (B) ICAM-1 western blot expression was normalised to α-tubulin with protein quantification (n=4) and cells were stained for ICAM-1 expression (green) with DAPI nuclear counterstaining (blue). Cells were imaged DMi8 microscope at 20x magnification. 1: LG; 2: DG; 3: DG+L; 4: DG+L+Ex(9-39). Each column represents mean ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni’s post-hoc test *P < 0.05, **P < 0.01.
Figure 3-24: Effect of high glucose macrophage-conditioned media on murine cardiac endothelial cell sirtuin-1 protein expression and the influence of liraglutide. Representative sirtuin-1 protein expression in cardiac endothelial cells treated with macrophage-conditioned media; LG: L-glucose (25mmol/L), DG: D-glucose (25mmol/L), DG+L: D-glucose+liraglutide (10nmol/L), DG+L+Ex(9-39): D-glucose+liraglutide+exendin(9-39) 100nmol/L for 24h. (A-B) Sirtuin-1 western blot expression was normalised to α-tubulin with protein quantification by image J software (n=4); and (C) cells were stained for sirtuin-1 expression (green) with DAPI nuclear counterstaining (blue) and were imaged using DMi8 microscope at 20x magnification. 1: LG; 2: DG; 3: DG+L; 4: DG+L+Ex(9-39). Each column represents mean ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni’s post-hoc test. *P < 0.05.
Figure 3-25: Effect of high glucose macrophage-conditioned media on cardiac mouse endothelial cells tight junction Zonula occludens-1 (ZO-1) protein expression and the influence of liraglutide. (A) Representative Western blot for ZO-1 in cardiac endothelial cells treated with macrophage-conditioned media; LG: L-glucose (25mmol/L), DG: D-glucose (25mmol/L), DG+L: D-glucose+liraglutide (10nmol/L), DG+L+Ex(9-39): D-glucose+liraglutide+exendin(9-39) 100nmol/L for 24h; blots were normalised to α-tubulin. (B) ZO-1 western blotting protein quantification by image J software (n=4). (C) Cardiac endothelial cells stained for ZO-1 expression (red) with DAPI nuclear counterstaining were imaged using DMi8 microscope at 20x magnification. 1: LG; 2: DG; 3: DG+L; 4: DG+L+Ex(9-39). Each column represents mean ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni’s post-hoc test. *P < 0.05; ***P < 0.001.
3.4 Discussion

Recently, leukocytes, particularly macrophages, have become an increasing target of investigation further to their emerging pivotal role in development of cardiac dysfunction and heart failure associated with diabetes (Bajpai and Tilley, 2018). In this chapter, we demonstrated that liraglutide may exert anti-inflammatory effects on macrophages under high glucose conditions. Cytokine-rich high glucose conditioned media induced cardiac fibroblasts differentiation and endothelial cells dysfunction via paracrine signalling pathways, both of which were modulated by liraglutide treatment resulting in improved cell function. Taken together, these data support our hypothesis that GLP-1 signalling exerts cardioprotective effects in diabetes by altering macrophage actions.
We particularly focussed on the influence of GLP-1 agonists on cell-cell communication with specific relevance to inflammation-mediated cardiac remodelling and paracrine effects of infiltrating inflammatory cells on cardiac fibrosis and endothelial dysfunction through secretion of pro-inflammatory cytokines and chemokines (Fountoulaki et al, 2015). Indeed, direct treatment of fibroblasts with high glucose media is reported to induced fibroblast differentiation and collagen secretion (Shamhart et al., 2014), although it is likely that these effects would not be reversed by direct liraglutide treatment as cardiac fibroblasts do no express GLP-1Rs (Baggio et al., 2018). It is important to note the variation between western blot and immunocytochemistry results for GLP-1R expression in our study and further investigation in 3T3 fibroblasts, by for example, mass spectrometry would be beneficial in this regard.

Multiple evidence from the literature confirms the expression of the GLP-1R on macrophages (Arakawa et al., 2010; Tate et al., 2016) suggesting that liraglutide exerts its beneficial actions on fibroblasts via indirect effects on inflammatory cells, particularly macrophages, so may therefore attenuate inflammation in diabetes with secondary prevention of adverse ECM remodelling and endothelial dysfunction. However, 3T3 fibroblasts should be treated directly with high glucose 25nmol/L in the presence and absence of liraglutide 10nmol/L as a control to confirm first that they do not express GLP-1R and second effect of fibrosis that we got is mainly related to cytokines and chemokines secretome presented in high glucose conditioned media.

We initially conducted experiments with RAW 264.7 cells which were treated with high glucose in the presence and absence of GLP-1 agonists. Firstly, we confirmed expression of the GLP-1R in RAW 264.7 cells and investigated the effect of high glucose treatment in the presence or absence of exendin-4 (1nmol/L) or liraglutide (1nmol/L) on macrophages polarisation as indicated by cytokine and chemokine mRNA expression. As expected, intracellular mRNA expression of pro-inflammatory and anti-inflammatory cytokines and chemokines was altered by high glucose treatment in a time-dependent manner and modulated by exendin-4 and liraglutide. For example, it was demonstrated that liraglutide significantly reduced pro-inflammatory IL-1β expression at 24 and 72h when added to high glucose media, whilst increasing expression of anti-inflammatory, TGF-β at 72h, highlighting potentially beneficial actions of GLP-1R activation. The variable expression between time points may be explained by temporal regulation of cytokines, with some being induced at an earlier time point and subsequently downregulated with long-term
exposure leading to chronic inflammation activation. Indeed, macrophage interaction is known to be complex and dependent upon diverse signals and stimuli, so it is likely that our selected time points may not have completely reflected the high glucose-mediated inflammatory effect.

Based on previous studies, including from our laboratory, interaction between macrophages and fibroblasts is known to affect inflammation and to modulate synthesis of ECM proteins including collagen (Tate et al., 2016). Therefore, in order to investigate the influence of GLP-1 agonists on interaction between macrophages and fibroblasts, paracrine signalling experiments were performed by harvesting conditioned media from macrophages incubated in normal or high glucose in the presence or absence of exendin-4 or liraglutide which was used to treat cardiac fibroblasts followed by analysis of ECM protein expression. We decided to use similar protocol to what we had followed previously for BMDM (Tate et al., 2016); RAW cells were treated with high glucose in the presence or absence of liraglutide and/or exendin-4 for 72h with assessment of paracrine effects on primary murine cardiac fibroblasts. Interestingly, high glucose-treated macrophage-conditioned media increased expression of profibrotic genes in cardiac fibroblasts, such as α-SMA which is a marker of myofibroblast differentiation, which was attenuated by liraglutide but not exendin-4. These initial results therefore suggested that liraglutide may be more effective than exendin-4 in preventing fibroblast differentiation. However, as these RAW cell conditioned media experiments tended to yielded inconsistent data, for subsequent studies we tried to isolate primary murine bone marrow cells which were differentiated with macrophage colony stimulating factor following a previously established protocol; however, this proved to be largely unsuccessful as cells rarely survived in culture after isolation. As such, we decided to use immortalised murine BMDMs that were previously generated in the Wellcome-Wolfson Institute for Experimental Medicine and were kindly gifted by Prof José Bengoechea. In addition, as the use of primary cardiac fibroblasts was limited due to their low passage number and requirement to use multiple mouse hearts for each isolation per experiment, we decided to use the 3T3 fibroblast cell line for subsequent experiments to assess concentration-dependent effects of GLP-1 agonists on fibroblast differentiation.

Conditioned media was harvested from iBMDMs incubated in L-glucose or D-glucose in the presence or absence of exendin-4 or liraglutide at concentrations of 1 or 10nmol/L, with initial experiments indicating that 10nmol/L liraglutide had the highest efficacy to suppress fibroblast differentiation (α-SMA, COL1α2). Therefore, our
subsequent experiments were based on treating iBMDM with L-glucose, D-glucose with/without 10nmol/L liraglutide for 48h. Further studies following this protocol demonstrated that liraglutide altered inflammatory cytokine and chemokine expression through inhibition of high glucose-induced NF-κB nuclear translocation, which is known to induce inflammation (Filipov et al, 2005), whilst mechanistic analyses showed that liraglutide promoted increased cAMP generation, which mediated inhibition of NF-κB activation. This action was augmented in the presence of IBMX, as a phosphodiesterase inhibitor, and was counteracted by the adenylate cyclase inhibitor, MDL 12330A, suggesting that liraglutide mediates its anti-inflammatory effects via a cAMP/NF-κB dependent mechanism.

Hyperglycaemia in diabetes induces and promotes progression of cardiac and vascular disease through altering macrophage behaviour, increasing their migration (Omri et al., 2011), adhesion to cardiovascular tissues (Cheng et al., 2015) and secretion of inflammatory cytokines and chemokines (Wen et al., 2006), thereby driving tissue inflammation. Thus, inhibition of macrophage infiltration, adhesion and inflammatory cytokine secretion have emerged as targets to prevent cardiovascular manifestations in type 2 diabetes. Indeed, in our study, high glucose-treated macrophages demonstrated increased migration (Figure 3.15A), a central mechanism underlying macrophage infiltration to the diabetic heart, which was reversed by liraglutide. We also assessed effects on macrophage adhesion, as another key aspect of macrophage infiltration, and initially confirmed that high glucose treatment increased macrophage adhesion to the ECM protein, fibronectin, which was attenuated by liraglutide (Figure 3.16). Taken together, these results suggest that liraglutide may target macrophage behaviour suppressing their tissue adhesion, infiltration, and secretion of inflammatory mediators. This is consistent with previous studies which have reported that GLP-1 agonists modulate macrophage infiltration (Guo et al., 2016), alter their migration to the heart (Tate et al., 2016), vessel wall (Wang et al., 2014), and adipose tissue (Lee et al., 2012b), in addition to modulating macrophages adhesion (Arakawa et al., 2010), and specifically to the endothelium via high glucose induction of several transcription factors, such as NF-kB, and endothelial cell adhesion molecules (Piga et al., 2007).

Moreover, we aimed to identify key cytokines and chemokines secreted by macrophages after high glucose treatment which are modulated by liraglutide so may thereby be responsible for reducing adverse cardiovascular remodelling associated with experimental diabetes. We used a commercially-available proteome array to
assess cytokine and chemokine expression in macrophage-conditioned media in each experimental group, but due to limited resources one pooled sample from the 4 groups was analysed per membrane. Although the array is designed to identify 40 cytokines and chemokines, only 9 of them were detected clearly after treatment with macrophage-conditioned media. In this regard, it would be important to use concentrated conditioned media in the future that may facilitate additional proteins detection, together with ELISA analysis to validate and quantify expression of key target cytokines and chemokines.

It has been previously established that IL-1 plays a significant role in regulation of the inflammatory response involved in adverse cardiac remodelling, raising the possibility that the competitive IL-1 antagonist, IL-1Ra, may confer cardioprotective effects (Bujak and Frangogiannis, 2009). Indeed, increased IL-1Ra expression was detected in high glucose macrophage-conditioned media, which may reflect upregulation of anti-inflammatory signalling to counteract IL-1 activation during inflammation (Ballak et al., 2015), which appeared to be reduced by liraglutide. Similarly, protein expression of macrophage inflammatory protein-1α (MIP-1α) and MIP-1β were increased in high glucose conditioned media and normalised by liraglutide. This is consistent with previous reports of induction of MIP-1α and MIP-1β in murine myocardial infarction (Dewald et al., 2004), whilst MIP-1α inhibition reduced bleomycin-induced pulmonary fibrosis (Smith et al., 1994), and deletion of CCR1 receptor, which is primarily activated by MIP-1α, attenuated structural remodelling after myocardial infarction (Liehn et al., 2008), suggesting that liraglutide may play an equivalent pivotal role in inflammation and macrophage activation in experimental diabetes. Protein expression of the chemokine, CXCL10, was also found to be increased in high glucose, but was further induced by liraglutide. Indeed, it has been demonstrated that hyperglycaemia induces CXCL10 expression (Satrom et al., 2018), which paradoxically exerts anti-fibrotic actions in the infarcted myocardium (Saxena et al., 2014), suggesting that liraglutide may promote cardioprotection. Taken together, these data confirm the known remarkable diversity of macrophage cytokines and chemokines production which is associated with a complex interaction between external environmental stimuli and macrophage function, whilst indicating that such signalling pathways are likely to be modulated by liraglutide.

Our paracrine signalling experiments were performed using iBMDM and 3T3 fibroblasts as a relevant model to interrogate the influence of liraglutide on indirect cellular communication as the likely mechanism underlying its cardioprotective
actions in diabetes. We initially confirmed previous reports (Passino et al., 2015) that media from high D-glucose treated macrophages modulates expression of profibrotic genes, α-SMA, CTGF, and procollagen 1α2 (Figure 3.17), in addition to MMP-2 and MMP-9 which regulate ECM turnover, and the intermediate filament proteins, fibronectin (Heling et al., 2000) and vimentin (Yi et al., 2014). Notably, these changes were all reversed by liraglutide and abrogated by exendin(9-39), suggesting that activation of GLP-1R mediated paracrine signalling underlies reduced myofibroblast differentiation in this setting. In the present study, we also demonstrated alteration of fibroblast connexins expression after exposure to high glucose compared to L-glucose macrophage-conditioned media (Figure 3.20), which was recovered by co-incubation with liraglutide. Connexins are key transmembrane gap junction proteins that control intercellular communication and ion transfer between adjacent cells, thereby mediating cardiac action potential, and maintaining electrical conduction and regular rhythm (Gaudesius et al., 2003), reduced expression of which has been observed in CHF patients in association with pro-arrhythmic conduction (Burstein and Nattel, 2008). Our data therefore suggest that reduced inflammation in response to liraglutide in experimental diabetes may lead to decreased collagen deposition and fibrosis, at least partly due to attenuation of connexin expression. In fact, it has been established that matrix metalloproteinases (MMPs), particularly MMP-7, contribute to adverse post-myocardial infarction remodelling by increasing connexin-43 cleavage, whilst MMP-7 deletion improved connexin-43 expression and preserved myocardial conduction (Lindsey et al., 2006).

As Ca\textsuperscript{2+} signalling plays a pivotal role in control of cell function and maintenance of intercellular communication, we also investigated whether macrophage paracrine signalling alters Ca\textsuperscript{2+} signalling in 3T3 fibroblasts and the influence of liraglutide. Notably, it has been established that Ca\textsuperscript{2+} signalling not only mediates cell growth and differentiation, but also regulates cardiac fibroblast proliferation and ECM protein expression (Zhang et al., 2014), whilst MMP activation is Ca\textsuperscript{2+}-dependent. Consistent with these reports, our preliminary studies showed that fibroblasts treated with high glucose macrophage-conditioned media displayed increased free cytoplasmic Ca\textsuperscript{2+} in response to ionomycin and ATP in comparison to those treated with LG macrophage-conditioned media (Figure 3.21). Notably, CPA pre-treatment which inhibited Ca\textsuperscript{2+}-dependent ATPase activity of the Ca\textsuperscript{2+} pump in sarcoplasmic reticulum, did not abolish ionomycin or ATP responses indicating that the
sarcoplasmic reticulum may not be the only source of Ca\(^{2+}\) release in this model. Nonetheless, increased agonist-induced free cytoplasmic Ca\(^{2+}\) induced by high glucose macrophage-conditioned media in 3T3 fibroblasts was attenuated by liraglutide indicating modulation by GLP-1 paracrine signalling. Complementary analyses suggested that the observed effects of liraglutide on fibroblast Ca\(^{2+}\) signalling correlated with TRPV2 channel expression (Figure 3.22B) which was attenuated by liraglutide in a GLP-1R dependent manner. Notably, TRPV2-mediated Ca\(^{2+}\) channel activation is reported to regulate fibroblast differentiation, proliferation and ECM protein secretion, whilst inhibition of TRPC3 channels prevented atrial fibrillation mediated fibrosis (Harada et al., 2012).

To further interrogate effects of liraglutide on macrophage paracrine signalling, conditioned media experiments were also performed to assess interaction with endothelial cells as a key driver of pathological cardiac remodelling associated with diabetes. In this regard, inflammatory cytokines induced by diabetes, atherosclerosis and obesity are reported to play a central role in promoting endothelial dysfunction associated with increased expression ICAM-1, VCAM-1, and E-selectin (Hopps et al, 2011), whilst diabetes is linked with increased ET-1 production and impaired eNOS activation (Tabit et al., 2010). Consistent with previous findings, our results established upregulation of endothelial inflammatory adhesion markers, ICAM-1 and VCAM-1 (Figure 3.23 and 3.24), upon incubation with high glucose macrophage-conditioned media which was prevented by liraglutide in parallel with similar effects on the vasoconstrictor, endothelin-1 (ET-1) (Figure3.23C). Meanwhile, high glucose macrophage-conditioned media decreased the expression of vasoprotective factors, such as the vasodilator eNOS, and the vascular homeostatic mediator, sirtuin-1 (Figure 3.23D,F), which were rescued by liraglutide. In fact, sirtuin-1 is reported to ameliorate endothelial cell injury and to induce eNOS upregulation (Cheang et al., 2019), which may at least partly explain the observed vasoprotective actions of liraglutide in preventing endothelial dysfunction by inducing sirtuin-1 and thereby inhibiting NF-κB signalling (Kauppinen et al., 2013).

Finally, we performed complementary experiments to assess the effect of liraglutide-modulation of macrophage paracrine signalling on endothelial cell barrier function which is crucial to maintain endothelial cell structure, preserving cell-cell tight junctions and attachment to the basement membrane (Rodrigues and Granger, 2015), all of which are necessary to regulate endothelial cell permeability and to preserve vascular function. As expected, endothelial cells treated with high glucose
macrophage-conditioned media showed reduced barrier formation after seeding in an xCELLigence plate, as indicated by reduced cell index (Figure 3.27), together with decreased expression of the tight junction protein, ZO-1 (Figure 3.26), effects which were attenuated by liraglutide and abrogated by exendin(9-39). Indeed, diabetes is associated with reduced endothelial cell barrier function with impaired tight junction connections, thereby promoting development of endothelial dysfunction (Chistiakov et al, 2015), so liraglutide may also confer vasoprotective actions in this regard.

Taken together, the data presented in this chapter clearly indicate that the GLP-1 analogue, liraglutide, exerts anti-inflammatory effects on macrophages in response to high glucose which are mediated via specific modulation of cAMP/NF-κB signalling, as supported by published literature. Notably, NF-κB activity is considered as a central factor with regard to regulation of macrophage function and signalling, through reported effects on inflammatory mediators, cellular infiltration and adhesion, which all likely to adverse cardiovascular remodelling and dysfunction in diabetes. Our research group has reported previously that exendin-4 exerts cardioprotective actions by modulating macrophages actions which has now been extended to show that liraglutide modulates macrophage intracellular and paracrine signalling with both fibroblasts and endothelial cells in experimental diabetes which are likely to at least partly underlie the reported cardioprotective actions in this setting. Although the specific mechanisms are undoubtedly complex and interrelated, it therefore appears that liraglutide exerts its beneficial effects in diabetes not only by blood glucose regulation but also via its direct actions on macrophages.
4 Development of cell-targeted GLP-1 peptides with specific affinity for macrophages

4.1 Introduction

The role of inflammation in diabetic cardiomyopathy development is well established, with macrophages appearing to play a particularly pivotal role in mediating cardiac inflammation. Therefore, it seems logical to suggest that selective targeting of macrophage signalling may represent an effective therapeutic approach against diabetic cardiomyopathy.

4.1.1 Targeted drug delivery

Targeting drug delivery modalities are considered as promising therapeutic candidates with several advantages, such as extended drug half-life, improved efficacy and decreased toxicity. Targeted drug delivery may be achieved by two approaches: passive and active strategies. Passive drug targeting is characterised by extravasation of modified drugs to the desired site in the presence of leaky microvasculature which is characteristic of inflamed tissues, whilst active targeting involves binding of drugs to receptors which are preferentially overexpressed at target sites (Galvin et al., 2012).

4.1.2 Receptor-based approach to target macrophages

Macrophages express a variety of receptors, such as the mannose receptor and folic acid receptor. Drugs or particles containing ligands for these receptors may therefore promote specific targeting of drug delivery to macrophages. In particular, mannose is widely used as a ligand for macrophage drug delivery due to the prevalence of mannose receptor expression on these cells where it is involved in phagocytosis (Taylor et al., 1992). The mannose receptor (CD206) itself is a C-type 175-kDa lectin protein that possesses a terminal sugar as mannose, glucose, and N-acetylg glucosamine (McGreal et al., 2004). It has been established that addition of mannose to PLGA nanoparticles increases their uptake by macrophages compared with unmodified nanoparticles (Nahar and Jain, 2009; Saraogi et al., 2011). Similarly, addition of mannose to lipid nanoparticles encapsulated with rifabutin increased their uptake by alveolar macrophages six-fold versus unmodified lipid nanoparticles after intravenous injection (Nimje et al., 2009). Furthermore, mannose-modified liposomes demonstrate preferentially enhanced cellular uptake by macrophages both in vitro and in vivo compared to non-ligand mannosylated liposomes (Chono et al., 2008).
Various studies have reported expression of folate receptor-β (FR-β) in activated macrophages which shows high affinity for folic acid. FR-β is upregulated in macrophages in rheumatoid arthritis and pulmonary fibrosis (Paulos et al., 2004; Nagai et al., 2010) whilst FR-β is reported to be expressed on tumour-associated macrophages. Notably, FR-β is able to recognise and internalise FITC-labelled folate, indicating potential use as a biomarker for macrophage targeting (Puig-Kröger et al., 2009). Furthermore, folate-modified liposomes were found to enhance macrophage uptake in comparison to non-conjugated liposomes, highlighting potential for targeting drug delivery to activated macrophages without affecting normal cells and tissues. Indeed, folate-conjugated liposomes have been successfully targeted to tumour-associated macrophages in a murine model of ovarian cancer (Turk et al., 2004) indicating potential for cell-selective drug delivery.

### 4.1.3 Nanocarrier-based macrophage targeting

Nanocarriers are used to deliver drugs to the desired site, which may improve therapeutic drug efficacy. However, therapeutic application of nanoparticles has been limited due to rapid recognition and clearance by the reticuloendothelial system, (Moghimi et al, 2001) which is problematic when extended duration of nanoparticles-loaded drug availability is needed. Nanoparticles are spherical solid particles around 100nm in diameter which can be prepared from either natural or synthetic polymers. Different drugs, such as hydrophobic and hydrophilic small peptides and vaccines, can be encapsulated inside the polymer or may be conjugated and adsorbed on to its surface. Importantly, nanoparticles made from poly (lactic-co-glycolic acid) (PLGA) are approved by the FDA due to their low toxicity and tissue biocompatibility (Mundargi et al., 2008). During polymerisation, monomeric units of lactic acid and glycolic acids are linked together by ester linkages to produce linear polyester products (Astete and Sabliov, 2006) as shown in Figure 4.1.

![Chemical Structure of Poly (Lactic-Coglycolic Acid) (PLGA) polymer](image)

**Figure 4-1:** Chemical Structure of Poly (Lactic-Coglycolic Acid) (PLGA) polymer.

(m: is the number of lactic acid units and n is number of glycolic acid units). Adapted from: (Tabatabaei Mirakabad et al., 2014)
Another important property of PLGA is that it is a biodegradable polymer due to its hydrolysis to lactic acid and glycolic acid monomers, which are subsequently metabolised via the Krebs cycle and eliminated from the body as water and carbon dioxide, resulting in minimal toxicity (Dinarvand et al., 2011) as shown in Figure 4.2.

In general, the degradation time of PLGA depends on its copolymer ratio. As lactic acid is more hydrophobic than glycolic acid, lactic acid-rich PLGA nanoparticles are more hydrophobic and less hydrophilic than glycolic acid-rich nanoparticles, so they consequently absorb less water and degrade more slowly (Dinarvand et al., 2011).

![Figure 4-2: Hydrolysis of PLGA polymer to lactic acid and glycolic acid. Adapted from: (Tabatabaei Mirakabad et al., 2014)](image)

4.1.3.1 PLGA nanoparticles for drug delivery
Nanoparticle–based drug modification has many advantages for cancer drug delivery. For example, it promotes concentration of the anticancer agent at its site of action, thereby maximising its anti-tumour effect, whilst minimising drug exposure to healthy cells thus reducing unwanted drug toxicity. In addition, nanoparticles have capability to penetrate tissues and to reach key sites of action, such as the liver, lung and lymphatic system, thereby improving efficacy (Danhier et al., 2012).

With specific relevance to this thesis, dysregulated macrophage function is associated with many disease conditions, such as bronchial asthma, atherosclerosis and tuberculosis. It has been reported that rifampicin-loaded PLGA microspheres promoted efficient rifampcin delivery to alveolar macrophages (Makino et al., 2004), whilst inhalable PLGA nanoparticles have been developed and targeted to tubercle bacilli in alveolar macrophages. (Tomoda and Makino, 2007). Taken together, it appears that further development of PLGA nanoparticles as nanocarriers holds
significant therapeutic potential to enhance drug delivery and that direct targeting to macrophages may be particularly effective given their established role in several diseases. Whilst this may present an achievable strategy for treatment of cancer and other inflammatory conditions, delivery of drugs using PLGA polymers is associated with several disadvantages, such as high cost and low drug loading efficiency which may limit clinical application.

For advancement of GLP-1 drug delivery, in the present study we decided to modify GLP-1 analogues in order to promote specific targeting to macrophages, and thereby selectively promote GLP-1 actions on myocardial inflammation which appears to be the major mechanism by which they protect against adverse remodelling of the diabetic heart (Tate et al, 2016). To achieve this, we adopted two approaches: (1) addition of a mannose side-chain to exendin-4, and (2) attachment of GLP-1 analogues to modified PLGA nanoparticles. The effectiveness of these approaches was initially assessed by analysing effects of these modified GLP-1 peptides on binding efficiency and receptor activity in vitro.

4.1.4 GLP-1 receptor characteristics

4.1.4.1 G protein-coupled receptors (GPCRs) - structure and activation

G-protein coupled receptors (GPCRs) are the largest receptor superfamily in the body with at least 1,000 different members (Rosenbaum et al, 2009), which modulate sensory perception, neurotransmission, cell communication and other key physiological events (Saengsawang and Rasenick, 2016). To be classified as GPCRs, receptors should possess two key requirements: (1) having seven transmembrane α-helical domains with an extracellular amino-terminal segment and an intracellular carboxy-terminal segment (Rosenbaum et al, 2009; Oldham and Hamm, 2008) and (2) being coupled to a heterotrimeric guanosine nucleotide-binding protein or G-protein (composed of α-β-γ-subunit); hence, they are known as G-protein coupled receptors (Fredriksson et al., 2003).

GPCRs are mainly comprised of three major subfamilies, which include: (1) family A, which contains the largest GPCRs that are related to rhodopsin and β2 adrenergic receptors, (2) family B, which includes GPCRs related to glucagon receptors comprising approximately 20 different receptors of neuropeptides and peptide hormones, such as parathyroid hormone, calcitonin, glucagon and vasoactive intestinal peptide (VIP), and (3) family C, including GPCRs related to metabotropic neurotransmitters, like glutamine and γ-aminobutyric acid (GABA) receptors (Foord
et al., 2005). GPCRs can bind a plethora of different substances, such as peptides, non-peptide neurotransmitters, hormones, glycoproteins, lipids, nucleotides and proteases, to induce cell signalling (Hazell et al., 2012). The vast diversity of endogenous signals capable of binding to and activating GPCRs has made them the most successful group of proteins in terms of drug targets, with an estimated 50% of all available therapeutic drugs known to target GPCRs (Lagerstöm and Schiöth, 2008). The main mechanism of GPCR activation is through receipt of an external signal by binding of the ligand to the receptor. Notably, many GPCRs can stimulate multiple signalling pathways, whilst specific ligands may demonstrate different efficacies in relation to different pathways.

After ligand binding, the receptor changes conformation to convert the binding into an intracellular response through activation of a cascade of biochemical signals and molecular interactions (Rosenbaum et al., 2009) starting from activation of a secondary effector molecule, such as an enzyme which may promote or inhibit the synthesis of a variety of second messenger systems that control multiple intracellular pathways.

In their resting inactivated state, G proteins are assembled as a heterotrimer (α, β, γ) with GDP bound to the α subunit. Following ligand binding, a conformational change of the receptor occurs, which leads to exchange of GDP for GTP bound to the α-subunit (Hamm, 1998). Consequently, the G-protein subunits activate effector molecules, such as adenyl cyclase, guanylyl cyclase, phospholipase A2, phospholipase C, phosphodiesterase and phosphoinositide 3-kinase (PI3K) to promote or inhibit the generation of different second messengers, such as cAMP, cGMP, arachidonic acid, phosphatidic acid, diacylglycerol, and inositol triphosphate (Cabrera-Vera et al., 2003). In addition, GPCRs can regulate the concentration of a variety of ions, such as calcium and potassium, by opening or closing of ion channels (Tuteja, 2009). Whilst GPCRs are recognised to possess similar structures and activation mechanisms, different G-protein subtypes are linked with multiple transduction pathways so can produce a wide variety of cellular responses (Rosenbaum et al., 2009).
4.1.4.2 Glucagon like peptide-1 receptor (GLP-1R)

Glucagon-like peptide-1 (GLP-1) exerts its actions through binding to the GLP-1 receptor (GLP-1R), which belongs to GPCR class B. This class of GPCRs also includes receptors of the glucagon/secretin/vasoactive intestinal peptide receptor subfamily (Brubaker and Drucker, 2002). The GLP-1R consists of 463 amino acids and contains eight hydrophobic domains, seven of which are transmembrane, in addition to one extracellular N-domain. The extracellular N-domain is comprised of two α-helix and four antiparallel β sheets, which is stabilised by the disulphide bonds between six cysteine residues (Willard and Sloop, 2012). GLP-1R activation occurs via a two domain binding model, whereby the C-terminus of the ligand binds to the N-terminus of the receptor for peptide recognition, and the N-terminus of the ligand interacts with the GLP-1R transmembrane domain for receptor conformational structural rearrangement (Hoare, 2005) (Figure 4.3). This conformational change elicits a shift in the intracellular receptor loops, which is important for subsequent G-protein recruitment and signal transduction stimulation (Willard & Sloop, 2012).

4.1.4.3 Signal transduction of GLP-1

GLP-1 has pleiotropic effects, with evidence for signalling via multiple G-protein-coupled pathways. After peptide binding, the GLP-1R undergoes conformational changes and G protein recruitment thereby initiating signal transduction which activates intrinsic guanine nucleotide exchange factor of the receptor to release GDP from Gαs. Gα dissociates from Gβγ and subsequently binds to GTP. This results in activation of downstream pathways in which GαGTP stimulates membrane adenylyl cyclase to generate the intracellular second messenger, cAMP from ATP (Koole et al., 2013). The GLP-1R signal transduction mechanism is illustrated in Figure 4.3.
cAMP is the main effector of GLP-1R signalling. It activates protein kinase A (PKA) and phosphoinositide 3-kinase (PI3K), which in turn activates multiple downstream signalling pathways, including the mitogen-associated protein kinase/extracellular signal-regulated kinase (MAPK/ERK; also known as Ras-Raf-MEK-ERK), and PI3K/protein kinase B (AKT) pathways, the latter of which is considered an important target of GLP-1 signalling as it regulates a variety of physiological responses (Athauda and Foltynie, 2016). In addition, PKA stimulates cAMP-responsive element binding-protein (CREB) which induces the insulin receptor substrate 2 (IRS2) promoter in β-cells, thereby increasing the expression of IRS2, which is involved in regulating β-cell growth and survival (Baggio and Drucker, 2007a). Ultimately, cAMP signalling is terminated by the action of phosphodiesterase.

Moreover, GLP-1R stimulation induces membrane depolarisation of β-cells through increasing Ca\textsuperscript{2+} influx by opening of voltage-dependent Ca\textsuperscript{2+}-channels, and inhibition of K\textsuperscript{+} channels resulting in insulin exocytosis from β-cells (Baggio & Drucker, 2007a). Upon receptor–ligand binding, this complex is established to show rapid internalisation, after which it is sorted to endosomes where it is capable of generating cAMP (Figure 4.4); in contrast, inhibition of receptor-ligand internalisation can
attenuate GLP-1 responses (Kuna et al., 2013). However, the precise mechanism of GLP-1R internalisation and cAMP generation is incompletely understood.

Aims of this chapter
The rationale for this study is based our previous discovery that the GLP-1 analogue, exendin-4, preferentially modulates cardiac inflammation and extracellular matrix remodelling indirectly by targeting macrophages, which are the preferential inflammatory cell underlying GLP-1 mediated cardioprotective effects (Tate et al., 2016). Parallel in vitro studies found that exendin-4 altered macrophages response genes expression and paracrine signalling which was able to indirectly modify differentiation of cardiac fibroblasts, which do not express the GLP-1 receptor (Tate et al., 2016). Thus, the aim of this chapter is to generate modified GLP-1 peptides with high affinity for macrophages, whilst retaining GLP-1R binding affinity and capacity to modulate macrophage inflammatory signalling, which may ultimately be applied to reduce CHF progression in diabetes. GLP-1R affinity, efficiency and receptor activity of modified peptides were specifically assessed by measuring their ability to translocate the GLP-1R after stimulation, whilst also assessing their ability to stimulate cAMP production.
4.2 Materials and methods

4.2.1 Modification of exendin-4 by addition of mannose

We first designed a modified exendin-4 peptide by attachment of mannose with the aim of targeting selected macrophage surface antigens e.g. F4/80, CD206. As macrophages express the mannose receptor, CD206, which recognises terminal mannose, we hypothesised that addition of mannose to exendin-4 would increase its macrophage selectivity thus promoting binding with high affinity to the cell surface leaving the C-terminal domain free to activate the GLP-1R. Addition of mannose to exendin-4 was out-sourced to Creative Peptides (NY, USA). Initial discussions with the biochemist from Creative Peptides advised that either serine or threonine amino acid residues of exendin-4 could be conjugated with mannose. Although we would have preferred to design a series of mannose-modified peptides based on both exendin-4 and/or liraglutide, the high price (~$2,000 for 1mg) dictated that we start with a single peptide modified at a single position. Further to extensive literature searching, it was decided to investigate addition of mannose to position 11 of exendin-4 in the first instance as this was the least likely to cause significant alteration of peptide receptor binding affinity and activity (Adelhorst, et al, 1994). Consequently, mannose sugar was added to exendin-4 at serine residue position 11, as indicated in Figure 4.5.

![Figure 4-5: Exendin-4 peptide after addition of mannose sugar. Because macrophages express mannose receptor, thereby, addition of mannose to exendin-4 may promote macrophages specific targeting](image)

(A) Mannose structure, (B) Exendin-4 modified peptide with mannose at position 11.

4.2.2 Modification of peptides by attachment to PLGA nanoparticles

Further to the established phagocytic actions of macrophages and their ability to easily take up foreign particles, we next explored nanoparticles as a potential opportunity for selective inflammatory cell targeting of GLP-1 peptides, thereby improving drug therapeutic efficacy. These studies were performed in collaboration with Prof. Christopher Scott and Mr Adam Leach from the Centre for Cancer Research and Cell Biology (CCRCB), Queen’s University Belfast. As this approach was much less expensive, we studied a range of PLGA nanoparticles and GLP-1 peptide attachment strategies, using both exendin-4 and liraglutide. As previously
discussed in section (4.1.4.2), the C-terminus of the GLP-1 peptide is crucial for receptor activation, whilst the N-terminus acts as an “affinity trap” for binding the peptide ligand to loop residues of the GLP-1R. Therefore, we chose to initially modify exendin-4 and liraglutide by addition of PLGA nanoparticles to the peptide N-terminus so that the C-terminus remained free to interact with the GLP-1R. For initial addition of PLGA nanoparticles to liraglutide, we tested two approaches. Firstly, for nanoparticles comprised of PLGA alone, liraglutide was adhered to hydrophobic patches on the surface of the PLGA nanoparticles through its hydrophobic palmitoyl side chain (Figure 4.6A). Secondly, we employed PLGA-NHS nanoparticles which possess the standard hydrophobic characteristics of PLGA with the addition of n-hydroxysuccinimide (NHS) esters on the nanoparticle surface. These NHS esters selectively react with free amines, of which liraglutide only possesses one at its N-terminus (Figure 4.6B). It was therefore rationalised that this modification should cause the N-terminus of liraglutide to conjugate to the surface of PLGA-NHS nanoparticles.

Exendin-4 was also modified by the addition of PLGA-NHS nanoparticles which became bound to its amine group after release of n-hydroxysuccinimide. However, unlike liraglutide, it is more difficult to predict the exact amine residue which reacts most readily with the PLGA nanoparticle NHS group as this is most likely dependent upon their pKas (see Figure 4.7).

4.2.2.1 Synthesis of PLGA-based nanoparticle emulsion
PLGA polymers (RG502H, 20mg; Sigma-Aldrich, UK) were initially dissolved in 1ml dichloromethane (DCM) before the solution was cooled on ice prior to dropwise
addition with stirring to an aqueous solution of polyvinyl alcohol (PVA) in 2-(N-morpholino) ethanesulfonic acid (MES) buffer (2.5%, pH 5, 7ml). The mixture was then sonicated on ice (3sec on, 2sec off at 50% amplitude) before being stirred overnight to allow evaporation of the organic solvent. To synthesise rhodamine-encapsulated nanoparticles, rhodamine 6G (100µl of 2 mg/ml solution; Sigma-Aldrich, UK) was added to the organic phase (dissolved polymers in 1ml DCM). PLGA-NHS nanoparticles were purchased from PolySciTech (Indiana, USA) and were prepared as a standard functionalised nanoparticle formulation by mixing 25% (by mass) of the functionalised PLGA with 75% un-functionalised PLGA 502h (Resomer® RG 502 H; Sigma-Aldrich, UK) before dissolving in DCM.

To synthesise rhodamine-encapsulated nanoparticles, rhodamine 6G (100µl of 2 mg/ml solution; Sigma-Aldrich, UK) was added to the organic phase (dissolved polymers in 1ml DCM). PLGA-NHS nanoparticles were purchased from PolySciTech (Indiana, USA) and were prepared as a standard functionalised nanoparticle formulation by mixing 25% (by mass) of the functionalised PLGA with 75% un-functionalised PLGA 502h (Resomer® RG 502 H; Sigma-Aldrich, UK) before dissolving in DCM.

Figure 4-7: Exendin-4 modification by PLGA-NHS nanoparticles

4.2.2.2 Nanoparticle washing
The nanoparticle suspension was split into 5 x 1.5ml tubes which were centrifuged (16,600rcf, 20min, 4°C) and the supernatant discarded. Each pellet was then resuspended with 1ml MES buffer for washing and the process was repeated 3 times prior to sonication (amplitude 35%, 1sec on, 1sec off). Thereafter, centrifugation and sonication were repeated a further 2 times.

4.2.2.3 Addition of liraglutide or exendin-4
Liraglutide or exendin-4 peptide (3.8ml of 10µmol/L) were added to 10mg PLGA nanoparticles, resuspended and stirred at 100rpm for at least 4h. The drug-nanoparticle suspension was then split into 1.5ml tubes and centrifuged (17,900rcf, 20min, 4°C) and the supernatant discarded. Each pellet was resuspended in 1ml PBS and washing was repeated 3 times. After the final wash the pellet was resuspended in 1ml water, then sonicated for homogenous mixing on ice before being quantified using a microplate reader, as described below.
4.2.2.4 Peptide-nanoparticle quantification
Nanoparticles–drug concentration was quantified using a micro BCA protein assay kit (ThermoFisher Scientific, UK). Drug concentrations were prepared in a range of 0.078-5.0µmol/L as standards. Following the manufacturer’s instructions, the three working reagents from the kit were mixed together at appropriate percentages before being added equally to the standards and samples. Using a multi-well plate reader to measure colorimetric changes at 562nm absorbance, the concentration of the unknown prepared liraglutide/exendin-4 nanoparticle samples was calculated from the curve generated from the standard samples.

4.2.3 Evaluation of modified GLP-1 peptides receptor binding affinity, and receptor activation

4.2.3.1 Redistribution assay
The redistribution assay monitors the cellular translocation of GFP-tagged receptor after exposure to drugs or other stimuli. Upon GLP-1R stimulation by a ligand, the receptor is internalised/endocytosed. Thus, this GLP-1R assay is designed to screen for agonist receptor stimulation which induces internalisation of the GLP1R. Ligands/compounds are assayed for their ability to induce GLP1R internalisation by spot detection imaging, as described below.

4.2.3.1.1 U2OS cell culture
U2OS cells are adherent epithelial cells derived from human osteosarcoma, which stably express human GLP-1R fused to the N-terminus of enhanced green fluorescent protein (EGFP). This cell line was originally purchased from ThermoFisher Scientific (UK) and kindly donated for use in this study by Dr Lisa Connolly (Institute of Global Food Security, Queen’s University Belfast). U2OS cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) with Glutamax, supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin and 0.5mg/ml G418 to maintain GLP-1R expression at 37°C in a 5% CO₂ humidified atmosphere. U2OS cells were maintained at a confluence of 70-90% and were passaged every 3-4 days at a ratio of 1:10.

4.2.3.1.2 U2OS cells treatment for redistribution assay
U2OS cells were seeded using seeding medium (DMEM supplemented with Glutamax, 1% penicillin-streptomycin, 0.5mg/ml G418 and 1% foetal bovine serum) into 96-well black cell culture plates at a density of 6,000 cells/well and incubated for 18-24h prior to starting the assay using prepared media (DMEM supplemented with
2mmol/L glutamine, 1% penicillin-streptomycin). Test compounds of GLP-1 (7-36), exendin-4, mannose-modified exendin-4, exendin-4 N-terminus PLGA nanoparticles, liraglutide, liraglutide-adsorbed PLGA nanoparticles, and liraglutide N-terminus nanoparticles were diluted in assay medium to a 2X final concentration in a range of 2.74x10^{-9} - 2x10^{-6} M. 2X prepared peptides (100µl) were then added to the appropriate wells of a cell plate in triplicate, which was incubated for 1h at 37°C, 5% CO₂, and 95% humidity. The assay medium was then gently decanted and 100µl of 10% formalin was added to each well as a fixing solution and the cell plate was incubated at room temperature for a further 20min. Following fixation, cells were washed 4 times with 200µl PBS per well. After washing, 100µl of 1µmol/L Hoechst Staining Solution (9.5ml PBS, 0.5ml Triton X-100, 1µl Hoechst stain) was added per well, after which the plate was sealed with a black plate sealer and covered with aluminium foil for incubation at room temperature for at least 30min before imaging or storage at 4°C for up to 3 days in the dark. Finally, the cell plate was imaged using a ThermoScientific Arrayscan HCS reader with a 20x objective and the filters set for the Hoechst stain. The spot detector application was used where the SpotCountPerObjective measurement was selected to quantify the captured data.

4.2.3.2 cAMP assay
Classical activation of the GLP-1R induces guanine nucleotide exchange factor intrinsic to the receptor to catalyse release of guanosine diphosphate (GDP) bound to Gα, which subsequently binds to guanosine triphosphate (GTP), leading to dissociation of Gα. The dissociated Gα activates adenylyl cyclase (AC) which catalyses conversion of ATP to cAMP. As such, cAMP generation may be used to assess basal GLP-1R activity and response to stimulation.

4.2.3.2.1 CHL cell culture
Chinese hamster lung fibroblasts (CHL cells; CCL-39) previously transfected with the human GLP-1R were kindly provided by Dr Brian Green (Institute of Global Food Security, Queen's University Belfast). CHL cells were cultured in 4.5g/L glucose DMEM with GLutamax (Gibco) containing 10% (v/v) foetal bovine serum, 1% (v/v) of (100U/ml penicillin, 0.1mg/ml streptomycin) and 1% gentamicin (G418). They were maintained in an incubator at 37°C in an atmosphere of 5% CO₂ at a confluence of 70%-90% and were split every 3-4 days at a ratio of 1:10.
4.2.3.2.2 Determination of cellular cAMP using cAMP assay kit

Cells were harvested with the aid of trypsin/EDTA (0.05% Gibco) and seeded in a 24-well plate at a density of 3.0x10^5 per well for 16h. To assess cAMP generation, cells were washed with ice cold HBS buffer: 20ml of 10x HBS salts (130mmol/L NaCl, 900μmol/L NaH₂PO₄•2H₂O, 800μmol/L MgSO₄•7H₂O, 5.4mmol/L KCl, 1.8mmol/L CaCl₂•2H₂O), 4ml of 50X HEPES (20mmol/L HEPES), and 4ml of 100X phenol red (2.5mmol/L phenol red) supplemented with D-glucose (5.6mmol/L) and adjusted to pH 7.4. The assay ligands studied were as follows: GLP-1(7-36), exendin-4, mannosе-exendin-4, exendin-NHS nanoparticles, liraglutide, and liraglutide–adsorbed nanoparticles. They were diluted in HBS buffer supplemented with 1mmol/L 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich, UK) as a cAMP phosphodiesterase inhibitor (to avoid cAMP breakdown) to the appropriate concentration range (1.11x10^-7-1x10^-6M). 30µmol/L forskolin (Sigma-Aldrich, UK), as an adenylyl cyclase activator, served as a positive control. Cells were incubated with 200µl of test solution for 20min at 37ºC before cAMP was measured using a cAMP ELISA kit (R&D Systems, Minneapolis, USA) in accordance with the manufacturer’s instructions. After 20min, the test solution was removed and cells washed with ice cold PBS before being resuspended in cell lysis buffer 5 (diluted 1:5), and centrifuged at 600g at 2-8ºC for 10 min to remove cellular debris, after which supernatants were aspirated and stored at -20ºC for later assay assessment. Briefly, 50µl primary antibody was added per well of a 96-well plate, except for the wells used to assess non-specific binding, and incubated for 1h on a microplate shaker. Meanwhile, a series of standards of known cAMP concentration (3.75-240pmol/L) were prepared. After incubation with primary antibody, each well was aspirated and washed four times with 200µl wash buffer before addition of 50µl cAMP conjugate to all wells. After 15min, 100µl of each of the cAMP standards and cell lysates were added to the appropriate wells in triplicate. The plate was then covered and incubated at room temperature for 2h on a microplate shaker, after which each well was aspirated and washed four times with washing buffer before addition of 200µl substrate solution per well. Finally, the plate was incubated on the benchtop for 30min, when 100µl stop solution was added to each well, and optical density measured at 450nm and 540nm using the FLUOstar Omega 37 microplate reader (BMG LABTECH, Germany).

4.2.4 Assessment of liraglutide-modified peptides macrophages specific affinity by immunocytochemistry

Immunocytochemistry staining was performed to confirm that the modified peptides specifically targeted macrophages. For these experiments, iBMDM cells were grown
on coverslips in 6-well plates and incubated with 1nmol/L GLP-1(7-36) as a positive control, together with unmodified liraglutide, liraglutide-NHS nanoparticles, and liraglutide-adsorbed nanoparticles for 1h. The media was then aspirated, and the cells washed with PBS before they were fixed with 4% paraformaldehyde for 20min at room temperature. Cells were again washed with PBS before being permeabilised for 10min at room temperature in PBS containing 0.2% Triton X-100. They were then blocked with PBS containing 0.1% Triton and 10% goat serum for 30min at room temperature prior to final washing with PBS and incubation overnight at 4°C with primary antibodies to CD45 (1:200; CAT # 550566, BD Pharmingen, UK) as a positive control for macrophages, and GLP-1-FITC (1:100; CAT# 15672, Biorbyt), which has cross-reactivity with liraglutide, to detect uptake of unmodified liraglutide and liraglutide-modified nanoparticles. The next day, cells were washed three times with PBS before being incubated with an appropriate secondary antibody in the dark for 1h. After washing with PBS, slides were mounted, and coverslips were fixed in place prior to storage at 4°C for later analysis.

**4.2.5 Assessment of liraglutide-modified peptides macrophage-specific affinity by live cell imaging.**

iBMDMs were seeded in a glass-bottomed black tissue culture plate with glass bottom at density of 10000 cell per well. To assess nanoparticle-mediated targeting, macrophages were either treated with PBS as a negative control, liraglutide 10nmol/L with GLP-1-FITC antibody (1:100; CAT# 15672, Biorbyt), liraglutide-PLGA rhodamine nanoparticles 10nmol/L, and blank rhodamine -PLGA nanoparticles. The plate was placed in an environmental chamber for acquisition at 37°C and 5% CO 2. After 10-15 min for lens adjustment, live cell images were captured using a Nikon Ti2 W1 Spinning Disk microscope system with a 40X objective.

**4.2.6 Evaluation of modified GLP-1 peptides specific affinity for macrophages by flow cytometry**

To quantify modified peptide binding to the macrophage GLP-1R, which reflects macrophage targeting, flow cytometry analysis was performed to detect modified peptides-GLP-1R complex, following the methods described in section 2.6.

**4.2.6.1 Assessment of mannose-exendin-4 GLP-1R affinity**

Affinity of mannose-modified exendin-4 for the GLP-1R was previously assessed using the redistribution and cAMP assays, as described in sections 4.2.3.1.2, 4.2.3.2.2 respectively. To assess specific binding to and/or internalisation of mannose-exendin-4- by macrophages, RAW cells were split in to 10 T25 flasks in
preparation for flow cytometry analysis. Next day, the cells were divided into the following groups: unstained, untreated, exendin-4 treated, and mannose-exendin-4 treated at concentrations of 1, 10, 100, and 1000nmol/L. Cells were then stained with exendin-4 FITC-conjugated antibody (NBP1-05179F, Novus) at a concentration of 1:100 prior to detection on a BD Accuri C6 Plus flow cytometer (BD Biosciences, UK) at the School of Pharmacy, Queen’s University Belfast.

4.2.6.2 Optimisation of anti GLP-1 antibody concentration for flow cytometry
Since there is no specific anti-liraglutide antibody commercially available, an anti-GLP-1 antibody was used further to its 97% sequence homology with liraglutide, enabling cross-reactivity to detect liraglutide and liraglutide nanoparticles by flow cytometry. In brief, cells were stained with GLP-1 FITC-conjugated antibody (CAT# 15672, Biorbyt) for 30min on ice. Cells were then washed 3 times in FACS Buffer, as previously described in section 2.6, and resuspended in 500µl FACS Buffer before being quantified on the Attune NxT Flow Cytometer (ThermoFisher Scientific, UK) and data analysed using FlowJo software.

4.2.6.3 iBMDM cell permeabilisation to detect intracellular GLP-1R
Since we did not observe a positive flow cytometry shift in response to GLP-1 peptide/nanoparticle treatment, we recognised that our target, the GLP-1R, is rapidly internalised after stimulation which may hinder antibody binding to the liraglutide-receptor complex and subsequent detection by flow cytometry. Notably, GLP-1R internalisation occurs rapidly after receptor stimulation and has been reported to reach a maximum within 1h (Figure 4.8) (Thompson et al, 2016). Therefore, we hypothesised that detection of intracellular GLP-1R-drug complexes may require cell permeabilisation before antibody staining. In this regard, saponin and tween-20 were optimised as permeabilising agents prior to flow cytometry analysis of GLP-1/FITC. iBMDM cells were washed and counted and studied in the following 3 groups: unstained, untreated, and 10nmol/L liraglutide-treated. All cells were fixed with 100µl 4% formaldehyde for 15min at 4°C before being washed 3 times with FACs containing 0.1% permeabilising agent and centrifuged at 400rcf for 5min. Cells were then permeabilised with either 0.2% saponin or 0.2% tween for 15 min at 4°C for optimisation before being washed again 3 times with FACs containing 0.1% permeabilising agent and centrifuged at 400rcf for 5min, and blocking with 5% BSA for 15min. Finally, the untreated and liraglutide-treated groups were stained with anti GLP-1 antibody (1:100) for 30-45min at 4°C in the dark, prior to washing and pellet resuspension in 500µl FACs for running on the flow cytometer.
4.2.6.4 iBMDM cell permeabilisation and staining with isotype controls to assess antibody specificity.

Isotype controls are used as negative references to help differentiate non-specific background signal from specific antibody signal. Due to the non-specific shift observed in the untreated group, FITC anti-GLP-1 antibody isotype controls were used to test GLP-1 antibody selectivity and specificity. For this purpose, cells were prepared, as described previously in section 4.2.5.3, and one group stained with anti-GLP-1 antibody and the other group with its isotype IgG control at the same concentration.

4.2.6.5 Evaluation of GLP-1 APC antibody staining efficacy

Due to nonspecific binding of FITC anti-GLP-1 antibody, we performed cells staining with a recommended GLP-1 antibody (Cat# NBP2-66869, Novus) conjugated to APC using a commercially available Lightning-Link® kit (Allophycocyanin antibody and protein labelling; Cat# SKU: 705-0030, Expedeon). Cells were fixed with 4% PFA, permeabilised with 0.2% saponin, and stained with anti-GLP-1 APC conjugated antibody.

4.2.6.6 Assessment of liraglutide nanoparticle affinity for macrophages

After testing the binding efficiency of liraglutide-modified nanoparticles to the GLP-1R, flow cytometry was performed to assess their affinity for macrophages compared with unmodified liraglutide. In this experiment we used liraglutide modified with

Figure 4-8: Time dependent stimulation of human GLP-1 receptor and receptor internalisation by GLP-1, compound 2 and compound B.

HEK293 cells overexpressing hGLP-1R showed receptor internalisation in a time dependent manner as assessed by ELISA (Thompson et al, 2016)
rhodamine nanoparticles that is detected in rhodamine positive shift on the FL-2 channel. iBMDM were divided in to the following 5 groups: (1) unstained, (2) untreated, (3) 10nmol/L liraglutide, (4) 10nmol/L liraglutide nanoparticles, and (5) blank nanoparticles group. After 1h, cells were collected and counted and 2x10^5 cells per group, washed with 1ml FACs buffer and centrifuged at 400g for 5min, after which the supernatant was discarded. To minimise nonspecific background fluorescence signal due to PFA, cells were then fixed with 100µl fixation buffer (Cat# 88-8824-00, eBioscience) for 30min at room temperature, before being thoroughly washed with 1ml of 1X permeabilisation buffer (Cat# 88-8824-00, eBioscience), and centrifuged at 400-600g for 5min. The obtained cell pellet was resuspended in 100µl of 1X permeabilisation buffer with the recommended GLP-1 antibody conjugated to APC. The APC-labelled GLP-1 antibody (1:100) was then used to detect intracellular liraglutide-GLP-1R complexes by incubation with treated and untreated cells for 40min protected from light at room temperature. Finally, the cells were washed twice with 1ml of 1X permeabilisation buffer and centrifuged at 400g for 5min before the cell pellet was resuspended in FACs buffer for flow cytometry analysis.

Further to reports that M1 proinflammatory macrophages can recognise nanoparticles more selectively than unstimulated macrophages (Bagalkot et al., 2015), and given that the M1 macrophage phenotype is the most likely target by which GLP-1 agonists modulate diabetic cardiac remodelling, we decided to focus on this aspect. iBMDMs were therefore activated with 100ng/mL LPS for 24h to induce an M1 phenotype (Bagalkot et al., 2015) or 20ng/mL IL-4 for 24 h to induce an M2 phenotype (Casella et al., 2016) prior to treatment with liraglutide 10nmol/L, liraglutide-rhodamine PLGA nanoparticles or blank rhodamine PLGA nanoparticles. Cells were then stained for the M1 macrophages surface marker, CD38 (Jablonski et al., 2015), and the M2 macrophage surface marker mannose-receptor CD206 (Jablonski et al., 2015). In addition, liraglutide treated groups were stained with GLP-1 APC conjugated antibody after cells permeabilisation.

4.2.6.7 Flow cytometry analysis of liraglutide and liraglutide nanoparticles to assess their affinity to murine-derived peritoneal inflammatory cells

Male C57BL/6 WT mice were purchased from Harlan UK and used at 12-16 weeks. The experiment was performed under project licence PPL2821 with authorisation from the Department of Health (Northern Ireland) and approval by Queen’s University Belfast Animal Welfare and Ethical Review Body.
For experiments, 12 mice were randomised into the following 4 groups (3 mice per group): (1) PBS control, (2) liraglutide-treated, (3) liraglutide-PLGA rhodamine nanoparticles, and (4) blank PLGA rhodamine nanoparticles, which were all stimulated with 500µg Escherichia coli LPS i.p. in PBS for 24h. The next day, mice were injected with PBS, blank nanoparticles, or liraglutide or liraglutide-PLGA rhodamine nanoparticles at 25nmol/kg (Synpeptide, Purity>90%), the concentration used in our previous in vivo studies (Robinson et al., 2015; Tate et al., 2016).

After 1h, animals were humanely sacrificed by CO₂ overdose and peritoneal cells were collected by injection of sterile ice-cold PBS into the peritoneal cavity using a 23G needle. After injection, the peritoneum of each mouse was gently massaged to dislodge any attached cells into the PBS solution before the fluid was re-aspirated to collect as many cells as possible. The resultant cell suspension was placed into tubes on ice prior to centrifugation at 1500rpm for 10min, after which the supernatant was discarded, and the cells re-suspended in FACs buffer for counting. From each mouse, 2.0x10⁵ cells were loaded per tube in 100µl FACs buffer (10% heat inactivated serum (HIS) in PBS; Gibco, UK) and either left unstained or stained with antibody cocktails as Fluorescence Minus One (FMO) per tube and left for 15min at room temperature. One tube of ultracomp beads (eBioscience) per antibody was also prepared to complete compensation. Information regarding antibody cocktails and dilutions of antibodies is presented in Table 4.1.

Cells were centrifuged at 600g for 5min at 4°C, and the supernatant discarded before cells were fixed with 100µl fixation buffer (eBioscience) and incubated overnight at 4°C. The next day, tubes were centrifuged at 600g for 5min at 4°C, the supernatants discarded, and the cells re-suspended with 100µl of 1X permeabilisation buffer (eBioscience) containing GLP-1 antibody conjugated to APC using the Lightning-Link® kit (Allophycocyanin antibody and protein labelling) kit. Cells were then incubated with GLP-1 antibody for 30min at room temperature before being washed two times with 1X permeabilisation buffer, fixed with 100µl fixation buffer, covered with tin foil and visualised on the FACS Canto II flow cytometer (BD Systems, UK).
Table 4-1: Antibodies used for flow cytometry to detect peritoneal cell population

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>Efluor450anti-Mouse cd11c</td>
<td>CloneN418</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Efluor506anti-Mouse cd11b</td>
<td>CloneM1/70</td>
<td>eBioscience</td>
</tr>
<tr>
<td>PerCypefluor 710anti-Mouse Ly6G(Gr-1)</td>
<td>Clone1A8</td>
<td>eBioscience</td>
</tr>
<tr>
<td>PECy7 anti-Mouse F4/80</td>
<td>CloneBM8</td>
<td>eBioscience</td>
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</tbody>
</table>

4.3 Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM) using GraphPad Prism7. Data from the redistribution assay were analysed by nonlinear regression followed by Sigmoidal, 4PL [where X is log(concentration)], whilst cAMP ELISA and flow cytometry data were analysed by one-way ANOVA with post-hoc Bonferroni correction to compare between three groups or more. Differences between groups were statistically significant when P<0.05.

4.4 Results

4.4.1 Effect of GLP-1 agonists and modified peptides on GLP-1R translocation

Initially, concentration-response curves for GLP-1R internalisation in U2OS GLP-1R-EGFP cells were generated using various concentrations (1.37-1000nmol/L) of GLP-1(7-36), exendin-4, mannose-modified exendin-4, and exendin-NHS nanoparticles. Whilst both GLP-1(7-36) and exendin-4 promoted dose-dependent GLP-1R internalisation, there was limited response to mannose-exendin-4 (Figure 4.9). In contrast, exendin-NHS nanoparticles caused significant GLP-1R internalisation, although this was less efficacious than unmodified exendin-4 (<100 maximal response; Figure 4.10). In addition, concentration response curves were generated for unmodified liraglutide and liraglutide–nanoparticles for comparison with exendin-4, using the same range of concentrations (1.37-1000nmol/L) to drive receptor internalisation (Figure 4.11). Both liraglutide-NHS nanoparticles and liraglutide-adsorbed nanoparticles produced similar GLP-1R internalisation to unmodified liraglutide, as indicated by the observed 100% maximal response, which was not statistically different between groups.
Figure 4-9: Effect of GLP-1, exendin-4, and mannose-exendin-4 on GLP-1R internalisation in U20S cells. (A) Concentration-response curve to GLP-1(7-36), exendin-4, and mannose-exendin-4 (1.3-1000nmol/L). The captured data were quantified by SpotCountPerObjective measurement and are presented as mean ± SEM, n=4. (B-E) representative images showing internalisation of GLP1R-EGFP stimulated with test compounds at 1µmol/L: (B) blank, (C) GLP-1(7-36), (D) exendin-4, (E) mannose-exendin-4. GLP-1R activation indicated in HCA micrographs by fluorescence (green) within nuclei (blue); arrows indicate GLP-1R-EGFP internalisation. Images are viewed at x20 objective magnification. Data were analysed by Nonlinear Regression followed by Sigmoidal, 4PL, X is log(concentration).
Figure 4-10: Effect of exendin-4, and exendin-4-NHS nanoparticles on GLP-1R internalisation in U20S cells. (A) Concentration-response curve for exendin-4, and exendin-NHS nanoparticles (1.3-1000nmol/L). The captured data were quantified by SpotCountPerObjective measurement and are presented as mean ± SEM, n=4. (B-D) representative images showing internalisation of GLP1R-EGFP stimulated with test compounds at 1µmol/L: (B) blank (C) exendin-4 (D) exendin-NHS nanoparticles. GLP-1R activation indicated in HCA micrographs by fluorescence (green) within nuclei (blue); arrows indicate GLP-1R-EGFP internalisation. Images are viewed at x20 objective magnification. Data were analysed by Nonlinear Regression followed by Sigmoidal, 4PL, X is log(concentration).
Figure 4-11: Effect of liraglutide and liraglutide nanoparticles on GLP-1R internalisation in U20S cells. (A) Concentration-response curves for liraglutide, liraglutide adsorbed nanoparticles, and liraglutide-NHS nanoparticles (1.3-1000nmol/L). The captured data were quantified by SpotCountPerObjective measurement and are presented as mean ± SEM, n=4. (B-D) Representative images showing internalisation of GLP1R-EGFP stimulated with test compounds at 1µmol/L: (B) blank (C) liraglutide (D) liraglutide-adsorbed nanoparticles (E) liraglutide-NHS nanoparticles. GLP-1R activation indicated in HCA micrographs by fluorescence (green) within nuclei (blue); arrows indicate GLP-1R-EGFP internalisation. Images are viewed at x20 objective magnification. Data were analysed by Nonlinear Regression followed by Sigmoidal, 4PL, X is log(concentration).
4.4.2 Stimulation of cAMP production by GLP-1 agonists and modified peptides

cAMP production by CHL cells was assessed by analysing %B/Bo values of tested peptides. B/Bo values were calculated as: data from optical density at 540nm were subtracted from those obtained at 450 nm, then divided by the optical density of the zero standard (Bo) and multiplied by 100 for conversion into percentage bound over un-bound (%B/Bo). The standard curve was calculated by plotting the calculated %B/Bo values of the standards against the known concentration of cAMP standards, and the B/Bo values of tested peptides were interpolated from the derived standard curve. Forskolin was used as a positive control, which, as expected, was found to significantly increase cAMP production compared to blank nanoparticle-treated CHL cells (P<0.001; Figure 4.12).

For each tested peptide, cAMP generation was found to be induced in a concentration dependent manner, with a statistically significant increase in cAMP production observed in response to both GLP-1(7-36) and exendin-4 when comparing concentrations of 1000 and 111.1nmol/L (P<0.01; Figure 4.12). In contrast, mannose–exendin-4 failed to increase cAMP generation in comparison to exendin-4 (P<0.001); for example, whilst 1000nmol/L exendin-4 produced ~50pmol/mL cAMP, the same concentration of mannose-exendin-4 produced <20pmol/mL cAMP, indicating that addition of mannose to exendin-4 impairs both GLP-1R binding and activation (Figure 4.12). On the other hand, exendin-4-NHS nanoparticles (1000nmol/L) induced significant cAMP generation versus mannose-exendin-4 (1000nmol/L). This confirms that addition of exendin-4 to PLGA nanoparticles does not affect its ability to induce GLP-1R translocation (Figure 4.10), and consequent cAMP generation (Figure 4.12). Similarly, liraglutide-adsorbed nanoparticles resulted in a marked increase in cAMP production which was greater than that observed in response to unmodified liraglutide at 1000nmol/L (P<0.05; Figure 4.13), which was abolished by the GLP-1R antagonist, exendin(9-39) at 10µmol/L. Taken together, these data suggest that adsorption of liraglutide to PLGA nanoparticles may promote its activity in a GLP-1R-dependent manner, whilst addition of exendin-4 to NHS nanoparticles does not and may even tend to reduce exendin-4 activity.
Figure 4-12: Induction of cAMP production by GLP-1 peptides and nanoparticles. Chinese hamster lung fibroblasts transfected with the human GLP-1R were incubated with different concentrations (111.1-1000nmol/L) of GLP-1(7-36), exendin-4, mannose-exendin-4, exendin-4-NHS nanoparticles, and blank nanoparticles, together with forskolin as a positive control. This representative blot is from three independent replicates n=3; each column represents mean ± SEM. cAMP values were analysed by one one-way ANOVA followed by Bonferroni post-hoc test. **P<0.01; ***P<0.001.

Figure 4-13: Liraglutide adsorbed PLGA nanoparticles stimulate the GLP-1R and induce cAMP. Chinese hamster lung fibroblasts transfected with the human GLP-1R were incubated with different concentrations (333.3 and 1000nmol/L) of liraglutide, liraglutide adsorbed nanoparticles, and blank nanoparticles, together with forskolin as a positive control. The representative blot is from five independent replicates (n=5); each column represents mean ± SEM. cAMP values were analysed by one one-way ANOVA followed by Bonferroni post-hoc test. *P<0.05; **P<0.01; ***P<0.001.
4.4.3 Immunostaining revealed uptake of PLGA nanoparticles by macrophages

Immunocytochemical analysis of iBMDM showed that both unmodified liraglutide and liraglutide nanoparticles were able to translocate to the GLP-1R, which upon binding is internalised to the cell cytoplasm and was thereby detected by GLP-1-FITC conjugated antibody (Figure 4.14). GLP-1R internalisation is signified by the green cytosol staining in panels B-E compared to the GLP-1 untreated group in panel A which represented the negative control. Complementary live cell imaging indicated the expected uptake of both liraglutide-PLGA nanoparticles and blank PLGA nanoparticles by iBMDM (Figure 4.15) further to their established phagocytic ability.

4.4.4 Flow cytometry analysis of in vitro macrophage targeting by liraglutide-PLGA nanoparticles

As a more sensitive measure, flow cytometry was also utilised to assess GLP-1R targeting in macrophages by modified GLP-1 peptides. Exendin-4 did not induce any positive flow cytometry shift in RAW cells (Fig 4.16A), whilst as expected, further to our previous data, addition of mannose did not induce any shift indicating that this modification impedes exendin-4 receptor interaction (Fig 4.16B). Surprisingly, however, exendin-4 also did not produce a positive shift in GLP-1R interaction at all different tested concentration in RAW cells (Figure 4.16B). Although RAW cells are known to express the GLP-1R, we decided to repeat these experiments in iBMDM cells (kindly donated by Dr Adrien Kissenpfennig, Centre of Experimental Medicine, Queen’s University Belfast), as due to their origin as an Abelson leukaemia virus transformed cell line derived from BALB/c mice, and iBMDM may more accurately represent primary macrophages. Indeed, we initially tried to isolate primary mouse bone marrow derived macrophages, but these cells did not survive in culture. However, flow cytometry analysis indicated that different concentrations of FITC-anti-GLP-1 antibody were also incapable of recognising and binding to liraglutide-treated immortalised bone marrow derived macrophages (Figure 4.17). As similar observations were made with both RAW cells and iBMDM, we concluded that it is likely to be due to rapid GLP-1R-liraglutide complex internalisation which impairs antibody binding to liraglutide, therefore necessitating prior cell permeabilisation in order to enhance liraglutide-antibody binding.
Figure 4-14: Representative images of GLP-1 peptide binding to iBMDM GLP-1R. iBMDM were treated with 10nmol/L GLP-1(7-36) as a positive control, liraglutide, liraglutide-NHS, or liraglutide-adsorbed nanoparticles for 1h. Cells were stained with CD45 as a positive control for macrophages (red) and FITC antiGLP-1 antibody to detect GLP-1R-peptide binding after drug-receptor interaction that is detected by drug-receptor complex internalisation (green). (A) Negative control, (B) GLP-1(7-36), (C) liraglutide, (D) liraglutide-NHS nanoparticles, (E) liraglutide–adsorbed nanoparticles. Cells were imaged using Nikon confocal April 12, 40X magnification.

Figure 4-15: Representative live cell images of iBMDM after treatment with liraglutide and nanoparticles. iBMDMs were treated with (A) PBS as negative control, (B) liraglutide 10nmol/L, (C) liraglutide-adsorbed nanoparticles 10nmol/L, and (D) blank nanoparticles. The plate was placed in an incubated chamber at 37°C and 5% CO₂ for imaging using a Nikon Ti2 W1 Spinning Disk microscope system at 40X magnification.
We therefore decided to fix treated cells prior to permeabilisation and analysis of GLP-1 antibody staining by flow cytometry as an alternate approach to detect the peptide-receptor complex. Given that our previous analysis of GLP-1R translocation had indicated that mannose-modified exendin-4 did not bind to and induce GLP-1R internalisation, it was not surprising that evaluation of mannose-exendin-4 treated fixed and permeabilised iBMDM by flow cytometry failed to detect bound GLP-1 peptide. However, iBMDM fixation using 4% paraformaldehyde (PFA) and permeabilisation with 0.2% saponin or 0.2% tween improved anti-GLP-1 antibody-liraglutide binding, as demonstrated in Figure 4.18, which clearly indicates a rightward shift upon treatment with unmodified liraglutide. Indeed, saponin induced a rightward shift on the BL-1 channel (Figure 4.18A) to a greater extent than tween-20 (Figure 4.18B) highlighting its suitability for use as a permeabilising agent to improve the flow cytometry signal. It is important to note that both untreated and liraglutide treated

![Flow cytometry analysis of exendin-4 and mannose-exendin-4 treated macrophages](image)

Figure 4-16: Flow cytometry analysis of exendin-4 and mannose-exendin-4 treated macrophages, RAW cells were treated with GLP-1 peptides at different concentrations for 1h before analysis of cell uptake using a FITC-anti-exendin antibody to recognise a positive shift in treated cells, indicative of increased cell fluorescence. Samples were tested using a BD Accuri instrument. Representative traces are shown for (A) exendin-4, and (B) mannose-exendin-4.
iBMDM induced a positive shift after the addition of FITC antiGLP-1 antibody compared to unstained cells (Figure 4.19). This may be due to antibody non-specificity which has been assessed for by the FITC antibody isotype that acts as a negative control to differentiate non-specific background signal from specific antibody signal. Ideally, the isotype control should not induce a positive shift compared to its primary antibody (Figure 4.20A). However, the results from our study showed that the isotype control induced the same degree of positive staining as its FITC conjugated antiGLP-1 antibody, indicating non-specificity of the FITC antiGLP-1 antibody (Figure 4.20B-D). In following experiments, cells were fixed with PFA and permeabilised with 0.2% saponin prior to staining with the APC conjugated antibody. As shown in Figure 4.21, this protocol still resulted in a rightward shift in the untreated group which may be explained by the addition of PFA as a fixation buffer causing induction of a nonspecific background signal during sample running in the flow cytometer (Spitzer et al, 2011). Therefore, further to difficulties encountered in obtaining a clean flow cytometry signal, we decided to use a commercial fixation-permeabilisation kit for future studies.

Thereafter, cells were fixed and permeabilised using a commercially available kit (eBioscience) prior to staining with APC conjugated anti-GLP-1 antibody to detect liraglutide on the RL-1 channel for comparison with rhodamine liraglutide PLGA nanoparticles detected on the BL-2 channel. Whilst unmodified liraglutide caused a clear rightward shift of the RL-1 channel signal, this was not observed with liraglutide-adsorbed or blank nanoparticles (Figure 4.22), indicating that unmodified liraglutide was taken up more by macrophages more effectively than liraglutide PLGA nanoparticles, suggesting that addition to PLGA nanoparticles may alter the ability of liraglutide to bind to the macrophage GLP-1R.
Figure 4-17: Optimisation of FITC anti-GLP-1 antibody concentration for flow cytometry. iBMDM were treated with liraglutide 10nmol/L for 1h, then stained with different concentrations of FITC anti-GLP-1 antibody. (A) antibody concentration 1:50, (B) antibody concentration 1:100, (C) antibody concentration 1:150, (D) antibody concentration 1:200.

Figure 4-18: Detection of liraglutide-receptor complex by flow cytometry after cell permeabilisation. iBMDM were fixed by 4% PFA, then permeabilised with (A) 0.2% tween or (B) 0.2% saponin before adding FITC anti-GLP-1 antibody to detect liraglutide receptor binding on the BL-1 channel.
Figure 4-19: Investigation of anti-GLP-1 antibody specificity by flow cytometry. iBMDM were divided in 3 groups: unstained, untreated, and liraglutide treated. Cells were fixed with 4% PFA, and permeabilised with 0.2% saponin before adding FITC anti-GLP-1 antibody to the untreated and liraglutide treated groups. (A) unstained, untreated cells, (B) unstained, liraglutide treated cells, (C) unstained, untreated, and liraglutide treated cells.

Figure 4-20: Investigation of anti-GLP-1 antibody specificity in iBMDM by flow cytometry using FITC anti-GLP-1 antibody and its isotype control. iBMDM were divided in to unstained, untreated and liraglutide treated groups. Cells were fixed and permeabilised using 4% PFA then stained with FITC anti-GLP-1 antibody for the liraglutide treated group and isotype staining for the untreated group. (A) ideal isotype control staining relative to unstained and antibody staining, (Bourdin, et al,2016), (B) unstained and isotype overlay groups, (C) unstained and liraglutide overlay groups, (D) liraglutide and isotype overlay groups.
4.4.5 Flow cytometry analysis of *in vivo* inflammatory cell targeting by liraglutide-PLGA nanoparticles

As M1 proinflammatory macrophages may be more inclined towards nanoparticle uptake, we first performed in vitro studies in which iBMDM were activated using LPS to assess whether macrophage phenotype influenced liraglutide and liraglutide nanoparticle interaction. Unfortunately, however, the flow cytometry data obtained for LPS activated macrophages was lost due to a problem with the machine whilst saving the results. Rather than repeating these experiments, we decided to move directly to *in vivo* testing of nanoparticle macrophages targeting. In these experiments, adult male mice received an intraperitoneal LPS injection in order to boost the inflammatory M1 macrophage population before they were treated with liraglutide and liraglutide-PLGA-rhodamine nanoparticles and subsequent collection of peritoneal inflammatory cells which were subjected to flow cytometry analysis. Inflammatory cell sub-populations were identified based on the expression of cell-specific surface markers to allow analysis of the effects of liraglutide and liraglutide nanoparticles on inflammatory cell GLP-1R binding and internalisation. Ly6G was used to specifically identify neutrophils, whilst F4/80 was used to identify macrophages, and CD11b was used to identify monocytes. Consistent with our *in vitro* data, we found that unmodified liraglutide induced a rightward shift in the flow cytometry signal in macrophages (Figure 4.23), neutrophils (Figure 4.24), and monocytes (Figure 4.25). Although quantification of these data (Figure 4.26) indicated that there were no statistically significant differences between groups, liraglutide-PLGA nanoparticles appeared to be less efficient in targeting macrophages and other inflammatory cells. Nonetheless, based on the findings of this experiment, it seems that adsorption to PGLA nanoparticles does not, as we hypothesised, increase liraglutide targeting to inflammatory cells, although we are unable to include whether this affected liraglutide-GLP-1R interaction. It is also interesting to note that in contrast to our anticipated finding that macrophages would be preferentially targeted by liraglutide in *vivo*, there was a tendency towards increased uptake by peritoneal monocytes in comparison to macrophages and neutrophils.
**Figure 4-21: Assessment of GLP-1 APC conjugated antibody staining efficacy**
iBMDM were divided in 3 groups: unstained, untreated, and liraglutide treated. Cells were fixed with 4% PFA, and permeabilised with 0.2% saponin before adding FITC anti-GLP-1 antibody to the untreated and liraglutide treated groups. (A) unstained, untreated cells, (B) unstained, liraglutide treated cells, (C) unstained, untreated, and liraglutide treated cells.

**Figure 4-22: Assessment of in vitro iBMDM targeting by liraglutide and liraglutide nanoparticles.** iBMDM were treated with 10nmol/L liraglutide, 10nmol/L liraglutide rhodamine-PLGA nanoparticles, or blank nanoparticles. The liraglutide treated group was stained with anti-GLP-1 APC conjugated antibody and detected on the RL-1 channel while rhodamine nanoparticles were positivity detected on the BL-2 channel. Surprisingly, these data indicate that liraglutide may target macrophages more effectively than liraglutide nanoparticles.
Figure 4.23 Assessment of *in vivo* macrophages targeting by liraglutide, liraglutide rhodamine-PLGA nanoparticles and blank nanoparticles in 12-week LPS-injected mice. After induction of inflammation by LPS for 24h, mice were injected with liraglutide 25nmol/kg, liraglutide-nanoparticles 25nmol/kg, or blank nanoparticles for 1h. Intraperitoneal lavage was then performed to collect inflammatory cells prior to assessment of liraglutide and nanoparticles targeting by flow cytometry. (A) liraglutide, (B) liraglutide PLGA nanoparticles, (C) blank nanoparticles.

Figure 4.24 Assessment of *in vivo* neutrophils targeting by liraglutide, liraglutide rhodamine-PLGA nanoparticles and blank nanoparticles in 12-week LPS-injected mice. After induction of inflammation by LPS for 24h, mice were injected with liraglutide 25nmol/kg, liraglutide-nanoparticles 25nmol/kg, or blank nanoparticles for 1h. Intraperitoneal lavage was then performed to collect inflammatory cells prior to assessment of liraglutide and nanoparticles targeting by flow cytometry. (A) liraglutide, (B) liraglutide PLGA nanoparticles, (C) blank nanoparticles.
Figure 4-25: Assessment of in vivo monocytes targeting by liraglutide, liraglutide rhodamine-PLGA nanoparticles and blank nanoparticles in 12-week LPS-injected mice. After induction of inflammation by LPS for 24h, mice were injected with liraglutide 25nmol/kg, liraglutide-nanoparticles 25nmol/kg, or blank nanoparticles for 1h. Intraperitoneal lavage was then performed to collect inflammatory cells prior to assessment of liraglutide and nanoparticles targeting by flow cytometry. (A) liraglutide, (B) liraglutide PLGA nanoparticles, (C) blank nanoparticles.

Figure 4-26: Geometric mean of fluorescence intensity for liraglutide, liraglutide-PLGA nanoparticle and blank nanoparticle uptake by peritoneal inflammatory cells in vivo. After mice had been stimulated with LPS for 24h, they were injected with liraglutide, liraglutide-nanoparticles, blank nanoparticles and PBS as a negative control for 1hr. Peritoneal lavage was then performed prior to assessment of tested compound uptake by flow cytometry (n=3).
4.5 Discussion

Inflammation has been widely implicated in the development and progression of diabetes and its associated cardiovascular complications (Reis et al., 2012). For example, upregulation of expression of inflammatory cytokines, IL-6, TNFα, TGF-β in the myocardium by infiltrating macrophages and monocytes is reported to activate transforming growth factor-β (TGF-β) in cardiac fibroblasts, thereby increasing fibrosis, collagen deposition, extracellular matrix synthesis, which ultimately drive adverse cardiac remodelling (Frati et al., 2017). Based on several previous reports, it seems that GLP-1 agonists confer cardioprotective effects against CHF progression in diabetes. Indeed, our group has shown that the GLP-1 mimetic, exendin-4, exerts specific benefits on post-MI remodelling in normoglycaemia via selective actions on inflammation and the ECM, which are characteristic of the diabetic heart (Robinson et al., 2015), whilst it confers similar cardioprotection in experimental diabetes, which are not seen in insulin-treated mice (Tate et al., 2016). In addition, data presented in this the previous chapter of this thesis found that conditioned media from iBMDM maintained in high glucose for 48h induced myofibroblast differentiation, as indicated by increased expression of α-SMA and CTGF, which was virtually abolished by pre-treatment with liraglutide. Taken together, these observations clearly suggest that the observed cardioprotective effects of GLP-1 agonists in diabetes are likely to at least partly occur by modulation of inflammatory cell activation and signalling.

In this chapter, we therefore tried to develop GLP-1 peptides with specific affinity for macrophages in order to maximise their evident anti-inflammatory actions. We first investigated whether addition of mannose which is known to bind to the mannose receptor (CD206), expressed on the surface of macrophages (Azad, 2014),and strategies that target this receptor may be effective in achieving macrophages drug delivery (Chaubey and Mishra, 2014). Thus, we tried to add mannose sugar to exendin-4 and assess the mannosylated peptide GLP-1R binding efficiency and activity. In fact, to assess modified peptides efficacy we depend on the criteria of GLP-1R ligand binding in which agonist binding induces GLP-1 receptor internalisation (Thompson et al, 2016) and cAMP generation (de Graaf et al., 2016). However, we found that mannose modification adversely altered exendin-4 efficacy, specifically causing impaired binding to the GLP-1R and reduced GLP-1R activation, as assessed by GLP-1R translocation (Figure 4.9) and cAMP generation (Figure 4.12) respectively. It is likely that these effects occurred due to the large molecular size of mannose sugar rendering ability for exendin-4 chain folding, therefore altered
receptor binding. Next, we tested whether attachment of exendin-4 or liraglutide to PLGA nanoparticles may be effective in promoting targeting to macrophages. Previous studies developed polymeric system as PLGA nanoparticles where GLP-1 agonists were added to the peptides for long term drug release (Kim and Kim, 2012). However, we tried to use PLGA nanoparticles to target drug delivery to macrophages (Spence et al., 2015) since they are capable to recognise foreign small substances. We started with exendin-4 by adding PLGA- NHS nanoparticles. This approach was also found to impair exendin-4 receptor interaction. In this regard, it may be that n-hydroxysuccinimide (NHS) ester PLGA nanoparticles did not react specifically with any single amine on exendin-4 but rather attached through random amine interaction, resulting in inconsistent formulation of the preparation which may explain the observed alteration in exendin-4 receptor binding affinity (Figure 4.10) and receptor activation (Figure 4.12). Nonetheless, we also tried to modulate liraglutide affinity for macrophages using the same approach by attachment to PLGA nanoparticles. Interestingly, the presence of a N-palmitoylglutamic acid fatty chain on liraglutide, which is not present on exendin-4, is likely to enhance adherence to the hydrophobic nanoparticle polymer. In addition, the n-hydroxysuccinimide (NHS) esters present on the surface of PLGA-NHS nanoparticles, which also demonstrate hydrophobic characteristics may selectively react with free amines on liraglutide at the N-terminus. However, it seems likely that any liraglutide which conjugates to the PLGA-NHS nanoparticle in this way also probably adheres to the surface of the particle via its palmitoyl tail. As result, in contrast to exendin-4, addition of liraglutide to PLGA-NHS nanoparticles did not impact upon the active binding site for GLP-1R interaction, as observed in U2OS cells overexpressing the human GLP1 receptor (Figure 4.11) and receptor activation in CHL cells overexpressing the GLP-1R (Figure 4.13). Having successfully identified a method for attachment of GLP-1 peptide to nanoparticles, we subsequently employed liraglutide nanoparticles for further studies towards establishing macrophage specificity. Whilst we found that addition of liraglutide to nanoparticles did not significantly impact upon its ability to bind to and activate the GLP-1R in macrophages in vitro as well as in vivo as it has been detected by flow cytometry. This modification was subsequently discovered to reduce liraglutide affinity for macrophages. It may be explained by rapid phagocytosis of PLGA nanoparticles before liraglutide has opportunity to binds to the surface GLP-1R, as that was clearly observed by live macrophages imaging (Figure 4.15). As a result, rapid internalisation may alter the ability of liraglutide to induce receptor activation in vitro and in vivo(Gustafson et al, 2015). It is also important to note that one of the
major reported disadvantages of PLGA nanoparticles is that they interact with cells in a non-specific manner which may cause accumulation of drug in non-target cells (Lu, Lv, & Le, 2019). Such properties may explain our in vivo results whereby the nanoparticles could have been rapidly cleared from the peritoneal cavity further to absorption by the lymphatic system, and mononuclear phagocytic system as liver, spleen. Therefore, resulting in reduced nanoparticle availability for uptake by peritoneal inflammatory cells (Gustafson et al., 2015) Nonetheless, PLGA nanoparticles addition altered liraglutide receptor binding in macrophages.

Taken together, addition of PLGA nanoparticles to liraglutide did not alter liraglutide - GLP-1R binding and activation as checked with U2OS cells and CHL cells respectively. Whilst it altered macrophages GLP-1R binding and activation. It therefore seems that further alternate modifications are required to effectively target liraglutide to macrophages, whilst maintaining or even augmenting its ability to activate GLP-1R signalling. Surface functionalization of PLGA nanoparticles by manose may become an approach to target the macrophages, reduce nonspecific tissue uptake, and to increase the chance for receptor binding before phagocytosis (Patel et al, 2018).

Other approaches may be to inhibit macrophage phagocytosis prior to treatment with liraglutide-PLGA nanoparticles or to encapsulate liraglutide in a pH-sensitive lipid capsule to promote specific release of the drug at the target site before phagocytosis by the cell. Indeed, it is possible to alter macrophage phagocytosis Targeted delivery of mannosylated-PLGA nanoparticles of antiretroviral drug to brain by blocking the clathrin pathway, specifically the clathrin heavy chain which plays an important role in macrophage endocytosis (El-Sayed & Harashima, 2013; Costa Verdera et al, 2017). In this regard, monodansylcadaverine (MDC) and cytochalasin are specific clathrin inhibitors (Kuhn et al., 2014) which have been shown to efficiently block clathrin-mediated endocytosis pathway. A further potential strategy for selective targeting of liraglutide to macrophages could be through modification of nanoparticles with a macrophage ligand-receptor. These may include addition of peptides, lectins or glycoproteins, which potentially increase macrophages specificity by taking advantage of both innate macrophage receptor expression and phagocytic processes (Figure 4.27).
Figure 4-27: Nanoparticles modification strategy to target macrophages. Adapted from (Chuang, Lin, A. Aljuffali, & Fang, 2015).
5 Investigation of potential effects of liraglutide on cardiac function and circulating inflammatory cell profile in type 2 diabetic patients

5.1 Introduction

Type 2 diabetes is associated with high risk of cardiovascular complications. Whilst tight control of blood glucose is the main goal to reduce the cardiovascular risk, benefits on macrovascular risk reduction are less certain. Emerging evidence indicates that GLP-1 agonists may provide extra benefits in addition to those associated with glycaemic control, which may reduce cardiovascular complications, such as hypertension, hyperlipidaemia and obesity, and consequent morbidity and mortality. In this regard, liraglutide is a GLP-1 analogue with established effects on blood glucose which also appears to reduce cardiovascular risk. Safety and efficacy of liraglutide were initially assessed by the Liraglutide Effect and Action in Diabetes (LEAD) trials, comprised of six randomised, multicentre, controlled double-blind studies, which were conducted in more than 40 countries and included 3,800 type 2 diabetic patients with poor glycaemic control and receiving conventional therapy. Efficacy and safety of liraglutide were compared with either placebo or comparator for a duration of 26 weeks, with the exception of LEAD-3 which was 52 weeks in duration as once-daily monotherapy.

In LEAD-1 and LEAD-2, the efficacy of liraglutide in lowering blood glucose levels was evaluated in combination with other antidiabetic drugs, including rosiglitazone, glimepiride, metformin, and insulin glargine. As sulfonylureas are widely used as first-line treatment for type 2 diabetes, mainly when metformin or thiazolidinediones are contraindicated or poorly tolerated, LEAD-1 specifically tested liraglutide in combination with glimepiride, with or without rosiglitazone, compared to placebo for 26 weeks to evaluate effects on glycaemic control and body weight. The findings of this trial showed that adding liraglutide or rosiglitazone to glimepride provided significantly better reduction in HbA1c than glimepride monotherapy, in which (1.2 mg or 1.8mg daily) liraglutide doses were superior for improving HbA1c. However, rosiglitazone addition unlike liraglutide, resulted in a significant increase in body weight (Marre et al., 2009). LEAD-2 was designed to investigate safety and efficacy of liraglutide addition to metformin in comparison to placebo or glimepride addition to metformin. It established that combination of liraglutide with metformin resulted in greater HbA1c reduction versus metformin monotherapy but was non-inferior to the
combination of glimepride and metformin in HbA1c reduction. However, a significant increase in body weight was reported in glimepride group compared to liraglutide treated groups (Nauck et al., 2009).

LEAD-3 evaluated liraglutide monotherapy for type 2 diabetes in comparison to glimepride monotherapy and reported greater reduction in HbA1c and body weight were recorded with liraglutide, although several patients in this group were discontinued due to vomiting as a reported adverse event of liraglutide (Garber et al., 2009). LEAD-4 demonstrated the benefits of liraglutide addition to metformin and rosiglitazone combination compared to placebo, reported specific improvement in glycaemic control, as indicated by decreased HbA1c, fasting and postprandial blood glucose level, which was associated with reduction in systolic blood pressure and body weight. However, gastrointestinal side effects were more frequently reported with liraglutide (Zinman et al., 2009).

As a follow-on to these trials, LEAD-5 established the effectiveness of liraglutide in combination with metformin and glimepride for metabolic control, as addition to standard insulin glargine therapy and/or placebo. Liraglutide was statistically superior to both placebo and insulin glargine in lowering blood glucose and reducing body weight in contrast to insulin glargine and placebo which induced weight gain (Russell-Jones et al., 2009). Finally, LEAD-6 compared liraglutide (1.8mg once daily) with exenatide (10µg twice daily) over 26 weeks added to standard antidiabetic drug therapy with metformin and/or sulfonylurea. Whilst exenatide and liraglutide were both well tolerated, incidence of minor hypoglycaemia was less frequent with liraglutide than exenatide, and the primary outcome of reduction of HbA1c was better with once-daily liraglutide versus twice-daily exenatide (Buse et al., 2009). Taken together, it appears that liraglutide is largely superior to other antidiabetic therapies with regard to blood glucose and body weight control, either as a monotherapy or combination therapy, and is generally safe with low incidence of hypoglycaemia, although transient nausea and vomiting are relatively common.
Further to establishment of its metabolic benefits and emerging cardiovascular actions in an experimental setting, the Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial was initiated in 2010 to investigate potent cardiovascular benefits in type 2 diabetic patients. LEADER was a placebo-controlled double blinded study in which patients were randomly assigned to receive once-daily 1.8mg liraglutide or placebo in addition to standard care with event-time and follow-up ranging from 42 to 60 months (Marso et al., 2016b). Cardiovascular outcomes were evaluated by 3 primary endpoint reductions defined as major adverse cardiovascular [CV] events [MACE]: first occurrence of CV death, nonfatal myocardial infarction or nonfatal stroke. Secondary cardiovascular outcomes were coronary revascularization, hospitalization due to unstable angina pectoris or heart failure, death from any cause, and presence of retinal and renal microvascular outcomes (Marso et al., 2016b). Notably, data from the LEADER trial highlighted significant reduction in its 3 primary endpoints with liraglutide compared with placebo, whilst mean HbA1c and all-cause mortality was also lower in the liraglutide treated group (Marso et al., 2016b). Interestingly, similar large-scale cardiovascular trials using exenatide (EXSCEL) and lixisenatide (ELIXA) failed to report cardiovascular benefit, suggesting that this could represent a class-specific effect of GLP-1 analogues (Hu, 2019).

Figure 5-1: Design of the LEAD trials incorporating the use of liraglutide as a monotherapy or in combination with other antidiabetic medications. Adapted from (Sethi et al., 2010)
In addition to the landmark LEADER trial, a complementary study was performed in type 2 diabetic Japanese patients undergoing peritoneal dialysis for 2 months to assess effects of liraglutide. Whilst the trial was primarily designed to determine safety and efficacy of liraglutide in these patients, it also included measurement of echocardiographic indices, such as left ventricular ejection fraction, left ventricular mass index and left ventricular eccentric hypertrophy, prior to dialysis and after 12 months. Interestingly, and consistent with LEADER, this trial reported significant benefits of liraglutide on blood glucose regulation and blood pressure which were associated with decreased left ventricular mass index at 12 months (Howell et al, 2019). As such, it appears that liraglutide treatment confers significant clinical benefit in type 2 diabetic patients both with regard to control of blood glucose level and reduction of cardiovascular risk. As discussed in the previous chapters of this thesis, we have reported that the GLP-1 mimetic, exendin-4, exerts direct cardioprotective actions independent of its metabolic effects. We established clear benefits of exendin-4 in experimental models of myocardial infarction and diabetes which were mediated via specific reduction of macrophage infiltration and attenuation of extracellular matrix remodelling and diastolic dysfunction, and occurred independently of parallel metabolic changes (Tate et al., 2016). In this regard, inflammation, which is emerging as a key driver of cardiac remodelling in diabetes and is preferentially altered by GLP-1 in the experimental setting, appears to represent an intriguing target. Therefore, the aim of this chapter was to assess the effects of liraglutide, which is reported to confer clinical cardiovascular benefit, on circulating inflammatory cell profiles and cardiac function in patients with type 2 diabetes.

Our hypothesis:
Null hypothesis (H0): Liraglutide is non-inferior to conventional antidiabetic therapies with regard to its anti-inflammatory effects and cardiovascular benefits.
Alternative hypothesis (H1): Liraglutide exerts anti-inflammatory effects which are associated with reduced cardiac remodelling and dysfunction compared to conventional antidiabetic drugs.
5.2 Materials and methods

5.2.1 Research design
The study was performed under the approved protocol (18/NI/0131) with authorisation from the Belfast Health and Social Care Trust and Queen’s University Belfast. Type 2 diabetic patients were recruited from the Royal Victoria Hospital Diabetes and Endocrinology clinic; they were 40-65 years of age receiving conventional antidiabetic medications or liraglutide as an additional treatment for ≥12 months. General inclusion criteria were HbA1c 7-10% (53-86mmol/mol) and BMI 30-40, whilst patients were excluded if they had received insulin, were recognised as having poor therapeutic compliance, were outside the stated age, BMI and HbA1c range, or had known history of cardiovascular disease. In addition, any patients who were recruited/consented and subsequently failed to attend for their single visit to the NI Clinical Research Facility were withdrawn from the study. Eligible patients who agreed to participate in the study were scheduled to visit the NI Clinical Research Facility on a single occasion, typically for 1-2 hours for blood collection (40ml for inflammatory cell profiling) and echocardiography analysis of cardiac function.

Patient recruitment numbers for this preliminary observational study were based on power calculations using published differences in echocardiographic function (e.g. left ventricular end-diastolic dimension) of 20% in diabetics versus non-diabetics with a confidence interval of 95% and 80% power. As such, it was decided that 20 patients were to be recruited to each group, although only a small proportion have are included in the preliminary analysis presented in this chapter.

5.2.2 Assessment of cardiac function by echocardiography
Echocardiography is considered as a safe, non-invasive and painless technique to examine cardiac structure and function on a serial basis. Indeed, the American Heart Association and the American College of Cardiology guidelines for the diagnosis and management of HF specifically highlight echocardiography as a useful investigation to diagnose and monitor patients with HF (Kirkpatrick et al., 2007). In the present study, all recruited patients were subjected to a single echocardiography analysis. Specifically, systolic and diastolic function were assessed by measuring:

1. Mitral valve blood flow was calculated by E/A ratio. This is represented by the peak velocity of early passive filling due to the pressure gradient (E wave) and the peak velocity of late active due to atrial contraction (A wave); i.e. the ratio of early to late filling (E/A). As, under normal physiological conditions most of
the blood fills the ventricle during early diastole, the E-wave is higher than the A-wave, with a typical ratio of 1.5 (Satpathy et al., 2006) whilst this may be altered in HF and is indicative of diastolic dysfunction.

2. Motion of the mitral valve annulus is calculated by the E/e´ ratio which is represented by the velocity of early mitral valve inflow Doppler signal (E) divided by the velocity of the early tissue Doppler signal (e´) (Marwick, 2015). In healthy individuals the E/e´ ratio is <8, whilst in the presence of diastolic dysfunction, the E wave increases and the annulus velocity e´ decreases, resulting in E/e´ elevation (Mitter et al, 2017).

3. Left atrium (LA) dimension: increased LA size is an adverse outcome seen in atrial fibrillation, hypertension, heart failure and cardiomyopathy, and is used as a reliable marker of diastolic dysfunction, due to elevated LA pressure and wall tension which is required to overwhelm increased intraventricular pressure (Lupu et al, 2014).

4. Left ventricular (LV) end-systolic dimension: measured at the end of systole i.e. the frame before mitral valve opening in which LV dimension is the smallest (Lang et al., 2006), and considered as a good indicator of systolic function.

5. Left ventricular (LV) end-diastolic dimension: measured at the end of diastole i.e. after closing of the mitral valve when LV dimension is the largest (Lang et al., 2006). As such, LV dimension is considered to provide a good estimation of ventricular size.

6. Interventricular septal diameter (IVSD) and left ventricular posterior wall (LVPW) thickness are used to assess left ventricular size and presence of hypertrophy as a marker of LV dysfunction and remodelling.

7. Left atrium volume index (LAVi) reflects LV changes mainly as a result of altered left ventricular filling pressure. LAVi is classified as normal: (28 mL/m²) or increased (mild: 29-33 mL/m²; moderate: 34-39 mL/m²; severe: >40 mL/m²) as an indicator of diastolic dysfunction and HF (Patel et al., 2015).

8. Left ventricular ejection fraction (LVEF) refers to the percentage of blood pumped from the filled left ventricle with each cardiac cycle. Normal LVEF is considered as ≥55%, whilst reduced LVEF is considered as <50% (Wagholikar et al., 2018).
Type 2 diabetic patients
40-65 years old, HbA1c 7-10% (53-86 mmol/mol), BMI 30-40

Patients receiving conventional therapy

Patients receiving liraglutide as an additional therapy for ≥12 months

Excluded from the study:
- outside the stated age, BMI and HbA1c range
- receiving insulin
- poor therapeutic compliance
- history of cardiovascular disease
- difficulties in adequate understanding of English

Recruited

20 patients receiving conventional therapy

20 patients receiving additional liraglutide therapy

Withdrawn from the study:
Fails to attend for single visit to the NI Clinical Research Facility

40 ml blood withdrawal for PBMC isolation

Echocardiography

Figure 5-2: Schematic diagram illustrating the clinical study design
5.2.3 Patient blood processing

5.2.3.1 Assessment of liraglutide treatment on inflammatory marker expression at baseline and after stimulation with LPS

To estimate the effect of liraglutide treatment on blood cell inflammatory marker expression, 1ml of the 40ml collected blood per patient was used to assess expression of cytokines and chemokines under both basal conditions and after stimulation. Of the 1ml volume, 500µl was used as a basal control, whilst the remaining 500µl was stimulated with 10ng/mL LPS (E.Coli, EMD Millipore, 0111:B4). Both samples were incubated for 6h at 37°C and 5% CO₂ prior to centrifugation at 1500rpm for 10 min. Plasma was collected from each sample and will be stored at -80°C until completion of the study for analysis of inflammatory marker expression in all patients by Luminex assay. Blood pellets were treated with 350µl Trizol (ThermoFisher, Cat 15596026) for isolation of RNA which was also stored at -80°C for future use.

5.2.3.2 Collection of patient plasma

The remainder of the collected whole blood (39ml) was centrifuged at 1500rpm for 10min at room temperature and the plasma transferred with a pipette to a sterile 15ml falcon tube which was further centrifugation at 1800rpm for 10min to remove any cells and debris. Aliquoted plasma samples were stored at -80°C for further analysis which may involve future assessment of, for example, systemic C-reactive protein or pentraxin-3, which reflect inflammation, and brain natriuretic peptide (BNP), which reflects cardiac dysfunction.

5.2.3.3 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated according to the protocol previously described in section 2.1.5.

5.2.3.4 Assessment of circulating inflammatory cell profiles in isolated PBMCs by flow cytometry

To specifically assess effects of liraglutide on circulating inflammatory cell compared to standard therapy, isolated PBMCs were stained for standard cell markers, CD3, CD16, CD11b, CD19, and CD56, to assess T-lymphocytes, neutrophils, monocytes/macrophages, B-lymphocytes and natural killer cell, respectively. Specific fluorophore antibodies are detailed in Table 5.1. 5.0X10⁵ PBMCs were loaded per tube in 100µl FACs buffer (10% heat inactivated serum (HIS) in PBS; Gibco, UK) and either left unstained or stained with antibody cocktails as Fluorescence Minus One
(FMO) per tube and left for 30min on ice. One tube of ultracomp beads (eBioscience) per antibody was also prepared to complete compensation. 1ml of FACs was then added to each tube to wash the cells before being centrifuged at 1200rpm for 5min prior to resuspension of the cell pellet in 100µl FACs which was covered with tinfoil and visualised on the FACS Canto II flow cytometer (BD Systems, UK). Flow cytometry data were analysed using Flowjo software.

Table 5-1: Antibodies used for inflammatory profiling by flow cytometry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Efluor450anti-human cd16</td>
<td>1:50</td>
<td>eBioscience</td>
</tr>
<tr>
<td>APC anti-human cd19</td>
<td>1:100</td>
<td>eBioscience</td>
</tr>
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<td>eBioscience</td>
</tr>
<tr>
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</tr>
<tr>
<td>PECy7 anti-human cd56</td>
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<td>eBioscience</td>
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</tbody>
</table>

5.2.4 Statistical analysis
Data are presented as mean ± standard error of the mean (SEM). Data were collated and statistically analysed using GraphPad Prism 7.0 (GraphPad, USA). An unpaired Student's t-test was used to compare data sets. Variants correlation was carried out using linear-regression analysis to calculate R-squared as the coefficient of determination. Multiple comparisons were carried out between data sets with a p value of <0.05 considered to be statistically significant.

5.3 Results
Of the total anticipated recruitment of 40 patients, 7 patients had participated in the study by October 2019 (4 patients receiving liraglutide and 3 patients receiving conventional oral glycaemic medications) at which time an interim analysis was performed for inclusion in this thesis. Unfortunately, recruitment was initially slow, which combined with withdrawal of several participants, resulted in low study numbers.
5.3.1 Clinical data and medications history

Patient medical and metabolic data were recorded, including age, HbA1c, and BMI, as specified by the inclusion criteria, in addition to blood pressure and circulating lipid profile (Table 5.2). Blood pressure values for all patients were within the normal range but tended to be lower in the liraglutide treated group which also appeared to show reduced plasma triglycerides; 3 of the 4 patients receiving liraglutide demonstrated triglyceride levels within the normal range ($\leq 1.8$mmol/L), whilst one patient on standard glycaemia medication displayed markedly elevated triglyceride concentration ($\geq 5.6$mmol/L). However, overall, no significant differences were detected between groups with regard to any of the recorded metabolic or clinical parameters (Figure 5.3), which may not be surprising considering the low patient numbers.

With regard to glycaemic medications, some patients received sodium glucose co-transporter inhibitors (e.g. empagliflozin; n=6), metformin (n=6), sulfonylurea derivative (e.g. gliclazide; n=2), a combination of sitagliptin (DPP-4 inhibitor) and metformin (Janumet®; n=1), thiazolidinedione derivative (pioglitazone, n=1), whilst 4 out of the 7 recruited patients received additional liraglutide. In addition to metabolic medications, cardiovascular medications were recorded. In this regard, the majority of patients received either angiotensin converting-enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs) due to their established cardioprotective and renoprotective benefits (Table 5.2).
Table 5-2: Demographic and clinical characteristics of all recruited patients

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Liraglutide group</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Age</td>
<td>56.7±2.85</td>
<td>54.75±1.65</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>63.3±6.44</td>
<td>68.25±4.71</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>32.6±0.72</td>
<td>33.675±1.33</td>
</tr>
<tr>
<td>BSA (m²)</td>
<td>2.02±0.16</td>
<td>2.50±0.10</td>
</tr>
<tr>
<td>Lipid profile (mmol/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>4.60±1.91</td>
<td>2.11±0.83</td>
</tr>
<tr>
<td>HDL</td>
<td>0.97±0.09</td>
<td>0.975±0.11</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td></td>
<td></td>
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<tr>
<td>Systolic BP</td>
<td>131.8±4.2</td>
<td>126.25±2.95</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>89.7±6.1</td>
<td>72.0±3.97</td>
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<tr>
<td>Antihypertensive medications</td>
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<tr>
<td>ACEIs (n)</td>
<td></td>
<td></td>
</tr>
<tr>
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<tr>
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<td>ARBS (n)</td>
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<td>No</td>
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<td>3</td>
</tr>
<tr>
<td>β-blockers (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>CCBs (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>2</td>
<td>3</td>
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<tr>
<td>Diuretics (n)</td>
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</tr>
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<td>1</td>
</tr>
<tr>
<td>No</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Values are mean±SEM.  
N: number of patients; BMI: body mass index; BP: blood pressure; TG: triglyceride; HDL: high density lipoprotein; β-blocker: beta-blockers; CCBs: calcium-channel blockers, ACEIs: angiotensin-converting enzyme inhibitors; ARBs: angiotensin-receptor blockers.
Figure 5-3: Clinical characteristics between control patients receiving conventional antidiabetic medication and patients receiving additional liraglutide. Each column represents mean±SEM. Data were analysed by one one-way ANOVA; p=NS.
5.3.2 Echocardiography data
Echocardiography was performed on a single occasion to assess cardiac structure and functional performance, with specific measurement of chamber dimensions, LV septal and posterior wall thickness, mitral valve inflow and ejection fraction, as important indicators of ventricular dysfunction. As mitral valve flow specifically reflects the pressure difference between atria and ventricle, any structural or functional abnormalities that may alter diastolic pressure will alter diastolic filling velocity, as indicated by E/A and E/E’. However, transmitral early flow velocity (E; Figure 5.4A), late flow velocity (A; Figure 5.4B), and E/A ratio (Figure 5.4C) remained similar between groups, whilst transmitral tissue flow (E/E’) and LAVi (measure specifically recommended by the American Society of Echocardiography which reflects changes in left ventricular diastolic filling pressure) were unaltered between patients receiving conventional antidiabetic medications in comparison to those receiving additional liraglutide (Figure 5.4D) and (Figure 5.4E), respectively.

Interestingly, however, liraglutide-treated patients demonstrated increased atrial wall dimension (P<0.05; Figure 5.5A), which is influenced by LV pressure and may thereby reflect altered diastolic function. There was also no evidence of chamber dilatation as assessed by left ventricular end-diastole dimension (Figure 5.5B). Similarly, left ventricular end-systole dimension remained similar between groups (Figure 5.5C), as did ejection fraction, values for which were all within the normal range for all patients (≥50%; Figure 5.6A), suggesting that systolic function was preserved and unaltered by liraglutide. It should be noted that only three ejection fraction values available in the liraglutide group as this index was not provided by the echocardiography technician for one patient.

Indeed, fractional shortening (FS), calculated as the difference between LVEDD and LVESD (Laddha et al., 2014) (Table 5.3), was also similar between groups (Figure 5.6B) and remained within the normal reference range for all patients (Table 5.3), (≥25%; except one patient receiving conventional antidiabetic medication who showed reduced FS of 21.3%), confirming absence of systolic dysfunction.
Figure 5-4: Echocardiographic measurements to assess left ventricular diastolic function and LV filling pressure.

Patients in the control group received conventional antidiabetic agents, whilst patients in the liraglutide group received additional liraglutide for at least 12 months. Doppler echocardiography was utilised to measure A: Early mitral flow velocity (E), B: Late mitral valve flow velocity (A); and C: E/A ratio, whilst tissue Doppler echocardiography quantified annular flow velocity to calculate D: E/E’ ratio. Left atrial size was measured as LA volume divided by body surface area (Left atrial volume index; E). Each column shows mean ± SEM. Data were analysed by one-way ANOVA; P=NS.
Measurement of posterior left ventricular wall thickness (LVPWT) and interventricular septal dimension (IVSD) is an important determinant of cardiac remodelling, and in particular, LV hypertrophy. In the present study, all patients showed normal values with no significant difference in IVSD and/or LVPWT between the two groups (Figure 5.7 A,B), whilst absence of LV hypertrophy due to e.g. longstanding hypertension or diastolic dysfunction, was confirmed by unaltered IVSD/LVPWT ratio (Table 5.4; Figure 5.7C).

An elevated IVSD/LVPWT ratio of ≥1.3 is commonly seen in patients with concentric LV hypertrophy, whilst a ratio of ≥1.5 may be more common in individuals with septal hypertrophy (Kansal et al, 1979), although the ratio remained within the normal range for all patients recruited to the current study. All patients’ echocardiographic indices are presented in Table 5.5.

<table>
<thead>
<tr>
<th>Control group</th>
<th>Liraglutide group</th>
<th>LVEDD (cm)</th>
<th>LVESD (cm)</th>
<th>Fractional shortening %</th>
</tr>
</thead>
<tbody>
<tr>
<td>•</td>
<td>5.1</td>
<td>3.7</td>
<td></td>
<td>27.45</td>
</tr>
<tr>
<td>•</td>
<td>4.7</td>
<td>3.7</td>
<td></td>
<td>21.28</td>
</tr>
<tr>
<td>•</td>
<td>4.0</td>
<td>2.5</td>
<td></td>
<td>37.50</td>
</tr>
<tr>
<td>•</td>
<td>4.8</td>
<td>2.7</td>
<td></td>
<td>43.75</td>
</tr>
<tr>
<td>•</td>
<td>5.4</td>
<td>3.5</td>
<td></td>
<td>35.19</td>
</tr>
<tr>
<td>•</td>
<td>4.5</td>
<td>2.8</td>
<td></td>
<td>37.78</td>
</tr>
<tr>
<td>•</td>
<td>4.9</td>
<td>3.2</td>
<td></td>
<td>34.69</td>
</tr>
</tbody>
</table>

Table 5-3: Calculation of LV fractional shortening as an indicator of systolic function
Figure 5-5: Echocardiographic measurements to assess chambers dimension. Patients in the control group received conventional antidiabetic agents, whilst patients in the liraglutide group received additional liraglutide for at least 12 months; A: Left atrium wall cavity; B: Left ventricular end-diastolic dimension; C: Left ventricular end-systolic dimension. Each column shows mean ± SEM. Data were analysed by one-way ANOVA followed by Bonferroni post-hoc test *P<0.05.

Figure 5-6: Echocardiographic measurements to assess left ventricular systolic function. Patients in the control group received conventional antidiabetic agents, whilst patients in the liraglutide group received additional liraglutide for at least 12 months. Echocardiography was performed to all patients to detect changes in LV cavity dimensions during systole to measure ejection fraction (A); during systole and diastole to calculate LV fractional shortening (B). Each column shows mean ± SEM. Data were analysed by one-way ANOVA.
Table 5-4: Calculation of IVSD/LVPWT ratio as an indicator of cardiac hypertrophy

<table>
<thead>
<tr>
<th>Control group</th>
<th>Liraglutide group</th>
<th>IVSD</th>
<th>LVPWT</th>
<th>IVSD/LVPWT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.1</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5</td>
<td>1.4</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.9</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.2</td>
<td>1.2</td>
<td>1.0</td>
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<tr>
<td></td>
<td></td>
<td>1.3</td>
<td>1.1</td>
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<td></td>
<td></td>
<td>1.36</td>
<td>1.35</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Figure 5-7: Echocardiographic measurements which are used to diagnose left ventricular hypertrophy. Patients in the control group received conventional antidiabetic agents, whilst patients in the liraglutide group received additional liraglutide for at least 12 months. Echocardiography was performed in all patients to measure A: Interventricular septal dimension (IVSD); B: Left ventricular posterior wall thickness (LVPWT); C: calculation of IVSD/LVPWT ratio to evaluate presence of left ventricular hypertrophy. Each column shows mean ± SEM. Data were analysed by one-way ANOVA.
Table 5-5: Baseline echocardiographic measurements in type 2 diabetic patients recruited to the study.

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Liraglutide group</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Left atrium, cm</td>
<td>3.83±0.12</td>
<td>4.35±0.13</td>
</tr>
<tr>
<td>LV end-systole, cm</td>
<td>3.30±0.40</td>
<td>3.05±0.18</td>
</tr>
<tr>
<td>LV end-diastole, cm</td>
<td>4.60±0.32</td>
<td>4.90±0.18</td>
</tr>
<tr>
<td>IVS D, cm</td>
<td>1.20±0.15</td>
<td>1.19±0.10</td>
</tr>
<tr>
<td>Posterior LV wall, cm</td>
<td>1.17±0.12</td>
<td>1.14±0.09</td>
</tr>
<tr>
<td>Mitral peak E, m/sec</td>
<td>0.72±0.11</td>
<td>0.72±0.13</td>
</tr>
<tr>
<td>Mitral peak A, m/sec</td>
<td>0.78±0.11</td>
<td>0.88±0.01</td>
</tr>
<tr>
<td>Mitral E:A ratio</td>
<td>0.92±0.05</td>
<td>0.94±0.11</td>
</tr>
<tr>
<td>E/E’ average</td>
<td>11.7±3.50</td>
<td>8.83±1.49</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>57.67±1.20</td>
<td>59.0±3.06</td>
</tr>
<tr>
<td>Indexed LA area, ml/m²</td>
<td>22.33±0.88</td>
<td>23.75±5.25</td>
</tr>
<tr>
<td>Fractional shortening%</td>
<td>28.74±4.73</td>
<td>37.85±2.08</td>
</tr>
<tr>
<td>IVSD/LVPWT</td>
<td>1.03±0.03</td>
<td>1.05±0.05</td>
</tr>
</tbody>
</table>

N: number of patients; LA: left atrium; LV: left ventricle; IVSD: interventricular septal dimension; LVPWT: Left ventricular posterior wall thickness; LVEF: Left ventricular ejection fraction.
5.3.3 Correlation between echocardiographic indices and metabolic profile

In order to assess whether metabolic changes in type 2 diabetic patients may influence cardiac function, a series of correlations were performed between metabolic measures and echocardiographic indices of diastolic and systolic function as E/A ratio, E/E’ ratio, LAVi and LVEF which are the most relevant indices to estimate cardiac function. Due to low numbers in each group (3 control, 4 liraglutide), it was not possible to perform between group comparison as this stage, although these key analyses will be performed in future when data collection has been completed.

Initial observations in 7 patients showed no apparent correlation between HbA1c and E/A ratio (P=0.555; R²=0.0739), E/E’ ratio (P=0.324; R²=0.1943), EF% (P=0.569; R²=0.08749) or LAVi (P=0.323; R²=0.1932) (Figure 5.8). However, BMI was better correlated with echocardiographic indices and demonstrated a significant negative relationship with LVEF (*P<0.05; R²=0.808), although this was not evident when compared with E/A ratio (P=0.345; R²=0.1784), E/E’ ratio (P=0.707; R²=0.031), and LAVi (P=0.069; R²=0.52) (Figure 5.9). It may be expected that alteration in lipid profile in type 2 diabetes may be associated with changes in cardiac indices. Indeed, plasma triglyceride appeared to show a negative correlation with E/A ratio (P=0.221; R²=0.281), E/E’ ratio (P=0.158; R²=0.354), and LAVi (P=0.329; R²=0.189), whilst HDL seemed to be positively correlated with E/A ratio (P=0.148; R²=0.3678), E/E’ ratio (P=0.0598; R²=0.54), and LAVi (P=0.102; R²=0.444) (Figure 5.10).

As blood pressure is known to be a major determinant of cardiac function, correlations were performed with the same echocardiography indices used for comparison with metabolic data. However, neither systolic nor diastolic blood pressure (both of which remained within the normal range in all 7 patients) showed any obvious correlation with cardiac function (Figure 5.11). Specifically, SBP and DBP showed mild positive association with LVEF% (P=0.392; R²=0.1863) and (P=0.315; R²=0.247), respectively and LAVi (P=0.1602; R²=0.352) and (P=0.546; R²=0.077), respectively, whilst SBP and DBP showed reduced correlation with E/A ratio (P=0.794; R²=0.0149) and (P=0.486; R²=0.101), respectively and E/E’ ratio (P=0.422; R²=0.132) and (P=0.991; R²=2.72X10⁻⁵), respectively.
Figure 5-8: Correlation of HbA1c to echocardiographic functional indices
Correlation of HbA1c to (A) E/A ratio; (B) E/E’ ratio; (C) ejection fraction % (EF); and (D) left atrial volume index (LAVi) in control patients (receiving conventional antidiabetic medication; grey dots) and patients receiving additional liraglutide; black dots.

Figure 5-9 Correlation of BMI to echocardiographic functional indices
Correlation of HbA1c to (A) E/A ratio; (B) E/E’ ratio; (C) ejection fraction % (EF); and (D) left atrial volume index (LAVi) in control patients (receiving conventional antidiabetic medication; grey dots) and patients receiving additional liraglutide; black dots.
Figure 5-10: Correlation of circulating lipid profiles to echocardiographic functional indices. Correlation of plasma triglyceride (TG) and high-density lipoprotein (HDL) to E/A ratio (A, E); E/E’ ratio (B, F); ejection fraction % (EF) (C, G); and left atrial volume index (LAVi) (D, H) in control patients (receiving conventional antidiabetic medication; grey dots) and patients receiving additional liraglutide; black dots.
Figure 5.11 Correlation of systolic and diastolic blood pressure to echocardiographic functional indices. Correlation of systolic (SBP) and diastolic (DBP) blood pressure to (A, E) E/A ratio; (B, F) E/E' ratio; (C, G) ejection fraction % (EF); and (D, H) left atrial volume index (LAVi) in control patients (receiving conventional antidiabetic medication; grey dots) and patients receiving additional liraglutide; black dots.
5.3.4 Inflammatory cell profiles in PBMCs isolated from type 2 diabetic patients

Flow cytometry analysis of PBMCs isolated from 6 of the 7 patients (PBMCs could not be collected from one control patient) revealed a clear positive shift in the CD3+ cell population, which reflect total T-lymphocytes, and a small signal from CD56+ natural killer cells and CD19+ B lymphocytes (Figure 5.12 and 5.13A-D). Similarly, myeloid cells, which include monocytes, macrophages, neutrophils, basophils, and eosinophils, were detected as a positive shift in CD11b+ and CD16+ staining in all diabetic patients. It should be noted that it is likely to mostly reflect the circulating monocyte population as CD11b+, whilst CD16/CD11b+ mostly reflect macrophages which are detected at low levels as they are mainly presented within tissues (Figure 5.12 and 5.13E). Although no significant differences in cell subtypes were detected between the two groups (Figure 5.14), there may be a tendency towards reduced circulating macrophages, B-cells and NK cells, and increased T-lymphocytes in liraglutide versus control patients, which may become more evident with higher patient numbers.
Figure 5-12 Flow cytometry analysis to detect different subsets of circulating immune cells in PBMCs. PBMCs were isolated from all patients and stained for (A) CD3+, T-lymphocytes; (B) CD56+, Natural killer-T cells (NKT); (C) T-cells (T-lymphocytes and NKT); (D) CD19+, B-lymphocytes; (E) CD11b+,CD16+, Myeloid cells (macrophages and monocytes). Data presented from one control (receiving conventional antidiabetic medication) and 2 patients receiving additional liraglutide.
Figure 5-13 Flow cytometry analysis to detect different subsets of circulating immune cells in PBMCs. PBMCs were isolated from all patients and stained for (A) CD3+, T-lymphocytes; (B) CD56+, Natural killer-T cells (NKT); (C) T-cells (T-lymphocytes and NKT); (D) CD19+, B-lymphocytes; (E) CD11b+, CD16+, Myeloid cells (macrophages and monocytes). Data presented from one control (receiving conventional antidiabetic medication) and 2 patients receiving additional liraglutide.
Correlation between circulating inflammatory cells and metabolic profile

As inflammation in diabetes may be linked to blood glucose levels and obesity, we performed comparisons between major circulating inflammatory cell populations and HbA1c and BMI values in the 6 patients from whom PBMCs were successfully isolated. Interestingly, HbA1c displayed significant association with B-cells (*P=0.0144; R²=0.811) (Figure 5.15E). However, there were no obvious relationships between these metabolic parameters and circulating inflammatory cells. Specifically, HbA1c levels showed weak association with circulating macrophages (P=0.931; R²=0.00217) (Figure 5.15A), monocytes (P=0.237; R²=0.3253) (Figure 5.15B), T-lymphocytes (P=0.831; R²=0.0127) (Figure 5.15C), and NKT-cells (P=0.256; R²=0.305) (Figure 5.15D). A similar pattern was observed upon comparison of BMI with macrophages (P=0.442; R²=0.154) (Figure 5.16A), monocytes (P=0.480; R²=0.131) (Figure 5.16B), T-lymphocytes (P=0.743; R²=0.0298) (Figure 5.16C), and NKT-cells (P=0.932; R²=0.0025) (Figure 5.16E), whilst B-cells showed highest correlation with BMI (P=0.152; R²=0.437), although this apparent negative relationship did not reach statistical significance (Figure 5.16E).

Figure 5-14: Frequency of inflammatory cells in PBMCs isolated from type 2 diabetic patients

Comparison of numbers of (A) macrophages, (B) monocytes, (C) T-lymphocytes, (D) B-cells, and (E) NKT cells in control patients (receiving conventional antidiabetic medication; n=2) and patients receiving additional liraglutide (n=4). Each column represents mean±SEM. Data were analysed by one-way ANOVA; p=NS.
Figure 5-15 Relationship between HbA1c and different inflammatory cells subsets. HbA1c level were correlated to (A) macrophages; (B) monocytes; (C) T-lymphocytes; (D) NKT-cells; and (E) B-lymphocytes in control patients (receiving conventional antidiabetic medication; grey dots) and patients receiving additional liraglutide; black dots.
Figure 5-16 Relationship between BMI and different inflammatory cells subsets. BMI values were correlated to (A) macrophages; (B) monocytes; (C) T-lymphocytes; (D) NKT-cells; and (E) B-lymphocytes in control patients (receiving conventional antidiabetic medication; grey dots) and patients receiving additional liraglutide; black dots.
5.4.1 Correlation between circulating inflammatory cells and cardiac function

Consistent with our hypothesis, we investigated the correlation between circulating inflammatory cells and cardiac functional indices. Interestingly, despite low patient numbers, increased macrophage frequency was positively and significantly correlated with early mitral flow velocity E (**P<0.01; R²=0.86)(Figure 5.17A), although macrophages only showed a mild correlation with late mitral flow velocity A (P=0.162; R²=0.422)(Figure 5.17B) and E/A ratio (P=0.3304; R²=0.2345)(Figure 5.17C), and limited association with E/E’ ratio (P=0.504; R²=0.118)(Figure 5.17D), LAVi (P=0.635; R²=0.0617) (Figure 5.17E) and EF (P=0.934; R²= 0.00196)(Figure 5.17F). Interestingly, monocytes, unlike macrophages, appeared to establish a negative correlation with LAVi (P=0.066; R²=0.6106)(Figure 5.18E), although monocytes showed no obvious link with early mitral flow velocity E (P=0.321; R²=0.242)(Figure 5.18A), late mitral flow velocity A (P=0.446; R²=0.151)(Figure 5.18B), E/A ratio (P=0.695; R²=0.04255)(Figure 5.18C), E/E’ ratio (P=0.572; R²=0.08613)(Figure 5.18D) or EF (P=0.596; R²=0.1)(Figure 5.18F).

Of the assessed echocardiographic indices, T-lymphocytes appeared to show closest negative correlation with early mitral valve flow velocity E (P=0.105; R²=0.5213)(Figure 5.19A), E/A (P=0.194; R²=0.378)(Figure 5.19C), and E/E’ (P=0.169; R²=0.4125)(Figure 5.19D), whilst late mitral valve flow velocity A (P=0.574; R²=0.08521)(Figure 5.19B), LAVi (P=0.452; R²=0.1472)(Figure 5.19E), and EF (P=0.341; R²=0.298)(Figure 5.19F) were poorly related. The only potential correlation for NKT cells was with E/E’ ratio (P=0.211; R²=0.3553) (Figure 5.20D), whilst all other assessed echocardiographic indices showed limited association (Figure 5.20).

Similarly, B-lymphocytes demonstrated only weak correlation with late mitral valve flow velocity A (P=0.250; R²=0.3103) (Figure 5.21B) and no obvious association with any of the other cardiac functional parameters (Figure 5.21).

Taken together, although largely preliminary in nature, these data indicate potential correlations between circulating inflammatory cell populations and measures of diastolic function, whilst systolic function, as assessed by LVEF %, seems to be less well associated. However, significant relationships between circulating inflammatory cells and cardiac function and the specific influence of liraglutide versus conventional antidiabetic medications cannot be assessed until increased numbers of patients have been recruited.
Figure 5-17 Correlation between macrophages and different echocardiographic indices. Frequency of circulating macrophages detected in PBMCs isolated from control patients (receiving conventional antidiabetic medication; grey dots) and patients receiving additional liraglutide (black dots) were correlated with (A) mitral valve early flow velocity E; (B) mitral valve late flow velocity A; (C) mitral valve E/A ratio; (D) E/E' ratio; (E) left atrial volume index (LAVi) and (F) ejection fraction (EF%).
Figure 5-18: Correlation between monocytes and different echocardiographic indices. Frequency of circulating monocytes detected in PBMCs isolated from control patients (receiving conventional antidiabetic medication; grey dots) and patients receiving additional liraglutide; (black dots) were correlated with (A) mitral valve early flow velocity E; (B) mitral valve late flow velocity A; (C) mitral valve E/A ratio; (D) E/E’ ratio; (E) left atrial volume index (LAVi) and (F) ejection fraction (EF%).
Figure 5-19 Correlation between T-lymphocytes and different echocardiographic indices. Frequency of circulating monocytes detected in PBMCs isolated from control patients (receiving conventional antidiabetic medication; grey dots) and patients receiving additional liraglutide; (black dots) were correlated with (A) mitral valve early flow velocity E; (B) mitral valve late flow velocity A; (C) mitral valve E/A ratio; (D) E/E’ ratio; (E) left atrial volume index (LAVi) and (F) ejection fraction (EF%).
Figure 5-20 Correlation between natural killers T-lymphocytes (NKT-cells) and different echocardiographic indices. Frequency of circulating NKT-cells detected in PBMCs isolated from control patients (receiving conventional antidiabetic medication; grey dots) and patients receiving additional liraglutide; (black dots) were correlated with (A) mitral valve early flow velocity E; (B) mitral valve late flow velocity A; (C) mitral valve E/A ratio; (D) E/E’ ratio; (E) left atrial volume index (LAVi) and (F) ejection fraction (EF%)
Figure 5.21: Correlation between B-lymphocytes (B-cells) and different echocardiographic indices. Frequency of circulating B-cells detected in PBMCs isolated from control patients (receiving conventional antidiabetic medication; grey dots) and patients receiving additional liraglutide; (black dots) were correlated with (A) mitral valve early flow velocity E; (B) mitral valve late flow velocity A; (C) mitral valve E/A ratio; (D) E/E' ratio; (E) left atrial volume index (LAVi) and (F) ejection fraction (EF%).
5.5 Discussion

Despite optimal blood glucose control in type 2 diabetes, development of cardiovascular complications, and particularly heart failure, remains a global challenge. Most patients develop sub-clinical diabetic cardiomyopathy even in the absence of coronary artery disease and hypertension that is characterised by diastolic dysfunction and raised morbidity and mortality risk, which is not significantly improved by tight glycaemic control, highlighting other mechanisms contributing to heart failure development. In this regard, inflammation (a characteristic feature of diabetes) and heart failure are strongly interconnected, with increasing myocardial inflammatory cell infiltration known to promote cytokine production, adverse cardiac remodelling, and heart failure (Riehle and Bauersachs, 2019). Accumulating evidence indicates that the GLP-1 analogue, liraglutide, acts on multiple pathways, thereby modulating cardiovascular risk factors, such as body weight, HbA1c (Ostawal et al., 2016) and lipid profile (Engelbrechtsen et al., 2017). It seems to particularly alter inflammatory cell activation and infiltration into the vascular wall and cardiac tissue, including specific modulation of cytokine production, as shown by our in vitro experiments described in previous chapters of this thesis. The aim of this chapter was therefore to build on our experimental data by investigating whether the apparently beneficial effects on liraglutide may be translated to the clinical setting. This was achieved by specifically evaluating the effects of long-term liraglutide treatment on inflammatory cell profile and cardiac structure and function in type 2 diabetic patients with comparison to conventional antidiabetic therapy.

Participants were selected on the basis of moderately controlled type 2 diabetes (HbA1c 7-10%) and moderate obesity (BMI 30-40 kg/m²), as this group is known to be prone to cardiac changes and inflammatory cell activation due to hyperglycaemia (Matheus et al., 2013) and weight gain (Nakayama and Wang, 2010). Importantly, only patients with no documented history of cardiovascular disease (defined as absence of heart failure and coronary artery disease) were included in the study in order that the specific influence of liraglutide on inflammation-associated sub-clinical cardiomyopathy could be interrogated. In addition, blood pressure, which has a major impact on cardiac structure and function, was well controlled in all patients through ACEIs, ARBs or CCBs, as antihypertensive medications. It is also important to note that the majority of recruited patients showed high plasma triglyceride concentration ≥2.3mmol/L, as none of them were receiving lipid-lowering drugs, although baseline
metabolic characteristics were not significantly different between groups at the time of initial data analysis. As previously discussed, diabetes-associated cardiac changes are typically characterised by diastolic dysfunction with preserved ejection fraction (Kenny and Abel, 2019), so often remain undiagnosed. Consequently, detection of echocardiographic changes offer potential for early diagnosis. In this regard, some studies have highlighted the presence of LV systolic strain alteration even with normal diastolic function and preserved ejection fraction (Ernande et al., 2011). In the present study, we therefore investigated whether liraglutide treatment for ≥12 months altered echocardiographic indices of LV structure and systolic and diastolic function. Whilst it is important to note the low numbers of patients included in our preliminary analysis, those treated with liraglutide appeared to show no improvement in diastolic function, as indicated by mitral valve E/A ratio, E/E’ ratio, or LAVi, which reflects increased LV filling pressure. However, it is interesting that of all the echocardiographic indices assessed, only left atrial wall cavity dimension was significantly changed between groups, showing an increase in liraglutide-treated patients (P<0.05), which would normally indicate diastolic dysfunction (Bouzas-Mosquera et al., 2011). As previously highlighted, it is difficult to draw any firm conclusions due to low patient numbers and incomplete matching of other medications, such as ACEIs or ARBs, which themselves have cardioprotective effects. A previous clinical study reported that chronic GLP-1 infusion improved LV function and increased LVEF in both diabetic and nondiabetic subjects with heart failure (Sokos et al., 2006b), although the reported effects of liraglutide on this index are heterogeneous. Some studies have demonstrated increased LVEF in patients with ST-segment elevation myocardial infarction (STEMI) (Chen et al., 2015b) and non ST- segment elevation myocardial infarction (non-STEMI) after liraglutide treatment (Chen et al., 2016) and in diabetic patients with reduced LV ejection (Arturi et al., 2017), whereas other studies have reported no change in LVEF with liraglutide therapy (Margulies et al., 2016). In the present study, additional liraglutide treatment was found to have no noticeable effect on cardiac function in type 2 diabetic patients without heart failure. Numerous studies have established that diabetes induces low-grade inflammation, thus activating monocytes and promoting their tissue infiltration. For example, high glucose-induced advanced glycation end product formation promotes monocyte migration and inflammatory marker expression, thereby contributing to cardiac complications (Goldin et al., 2006). Indeed, diabetes is associated with upregulation of immune cells, including lymphocytes and monocytes (Menart-Houtermans et al.,
2014), whilst T-lymphocytes have been specifically implicated in the pathogenesis of diabetes and insulin resistance (Xia et al., 2017) and associated cardiovascular disease (Zhao et al., 2014). Whilst initial analysis of our flow cytometry PBMC profiling data did not report any significant differences in circulating inflammatory cell populations between patients receiving conventional antidiabetic medications and those receiving additional liraglutide, numbers of CD3−CD56+ natural killer-T lymphocytes and CD19+ B-lymphocytes may have been reduced by liraglutide, despite the relatively low proportions of these cells known to be present in type 2 diabetic patients (Piątkiewicz et al., 2013; Zhou et al., 2018). It is also well established that diabetes promotes proinflammatory macrophage polarisation (Kanter et al., 2012) and activates secretion of proinflammatory cytokines. However, in the present study, macrophages (CD16+, CD11b+) were only recognised at very low frequency in the circulating PBMCs population, which is not surprising as there are mainly present in tissues further to monocyte migration and differentiation.

Previously, we reported in an experimental model of diabetes an improvement in diastolic function, as indicated by normalisation of E/A ratio and LV isovolumetric relaxation time, in mice treated with the GLP-1 analogue, exendin-4, compared to an insulin-treated control group with equivalent levels of glycaemia. Notably, this beneficial effect on cardiac function was associated with reduced myocardial macrophage infiltration with no changes in other inflammatory cell populations, including T cells and B cells. Exendin-4 was also found to alter secretion of a plethora of inflammatory cytokines and chemokines by macrophages in vitro, which we concluded was at least partly responsible for observed protection against adverse cardiac remodelling and dysfunction in diabetic mice (Tate et al., 2016). This intriguing finding formed the basis for the rationale for the clinical analyses described in the current chapter which were designed to further interrogate the cardiovascular benefits of liraglutide via correlation of circulating inflammatory cell profiles to cardiac structure and function in type 2 diabetic patients receiving long-term liraglutide therapy. Interestingly, although macrophages were found to only be present at low frequency within the circulating PBMC population, they showed a significant positive correlation with early mitral valve flow E, which was also apparent with both mitral valve E/A and E/E’ ratio, albeit with a much weaker relationship, suggesting that diastolic function may be correlated with macrophage numbers. Indeed, a previous small clinical study comparing 23 patients receiving liraglutide and 26 patients receiving placebo reported that liraglutide reduced early mitral valve flow E (Bizino et al., 2019), which is
consistent with the initial findings of our study indicating that circulating macrophages may influence or be influenced by cardiac diastolic function. Similarly, monocytes, which were detected at much higher levels than macrophages in the circulating PBMC population, displayed a weak correlation to mitral valve E/A ($R^2=0.04255$) and E/E' ($R^2=0.08613$) ratio, which may be reflective of their trafficking in to the dysfunctional myocardium. It would be interesting to further investigate this aspect in future tissue analysis (animal or human) of chemokine C-C motif ligand-2 (Ccl2) expression as an inducible monocytes-macrophage migration factor which may correlate with mitral valve flow velocity indices (Hulsmans et al., 2018). A previous study has also reported that myocardial T-lymphocyte infiltration is specifically associated with cardiac fibrosis and adverse remodelling in diabetic mice, which was attenuated by depletion of T-lymphocytes (Abdullah et al., 2016). Indeed, in the present study increased T-lymphocyte frequency appeared to be associated with reduction in early mitral valve flow velocity E, E/A, and E/E' ratio and increased LAVi, which would be consistent with this previous report in suggesting that T-lymphocytes play an important role in mediating cardiac inflammation and fibrosis in diabetes which could be subject to modulation by liraglutide.

Of all of the analysed circulating PBMC inflammatory cell populations in our study, natural killer T-lymphocytes displayed weakest correlation with echocardiographic functional indices, only indicating a weak correlation with E/E' ratio. Indeed, the specific role of NKT-cells role in cardiovascular disease development or prevention is unclear, with some studies reporting cardioprotective effects in preventing cardiac fibrosis via preventing fibroblast collagen accumulation (Strassheim et al., 2019), whilst other studies have indicated reduction of circulating NKT-cells in patients with cardiovascular disease is associated with increased tissue infiltration, suggesting that NKT-cells may contribute to inflammation and proinflammatory cytokine secretion (Van Puijvelde and Kuiper, 2017). In contrast, B-lymphocytes are known to play an important role in immune system homeostasis, whilst alteration in B-cell frequency and function may affect cardiac function and remodelling. We found that B-lymphocytes appeared to show a weak positive relationship with late mitral valve flow A, E/E’ ratio and LAVi, which may be consistent with their likely role in promoting cardiac remodelling. Specifically, B-lymphocytes have been reported to stimulate secretion of matrix metalloproteinase-9 (MMP-9), thereby inducing extracellular matrix (ECM) remodelling and fibrosis, this driving heart failure progression (Cordero-Reyes et al, 2013). In this regard, we may have expected to observe a positive correlation between patient inflammatory cell profiles and metabolic measurements,
such as BMI and HbA1c, which are known to be associated with inflammation and cardiovascular disease progression. However, our initial analysis of 6 patients from which PBMCs were successfully isolated demonstrated limited correlation between inflammatory cell sub-populations and metabolic parameters. Both displayed positive correlation with monocytes, and T-lymphocytes. However, of these, circulating monocytes appeared to show a weak positive correlation with HbA1c levels which is consistent with the suggestion that uncontrolled blood glucose and obesity may induce inflammation and immune activation (Dandona et al, 2004). In contrast, HbA1c and BMI displayed a negative relationship with NKT-cells and B-cells, whilst increased HbA1c were significantly associated with reduced numbers of B-cells (P<0.05), which play a pivotal role in adaptive immunity and regulation of immunoglobulin secretion. Indeed, it was previously established that alteration in the humoral immune system and immunoglobulin levels were negatively correlated to glycaemic control in diabetic patients with foot ulcers (Fejfarová et al., 2016), which may explain the observed negative relationship between HbA1c and B-cells in our study.

In summary, although different novel therapies have emerged to treat and/or prevent heart failure, it is still associated with poor prognosis. Thus, gaining detailed understanding of the apparently critical role of the immune system is likely to help in combat the disease, which is particularly prevalent in diabetes. In this regard, the findings presented in this chapter, although extremely preliminary, suggest that alteration of inflammatory cell profiles in diabetic patients may be associated with echocardiographic function, and thereby contribute to heart failure development and progression, thus highlighting selective targeting of inflammatory cells as a promising therapeutic strategy. In this regard, our in vitro studies described earlier in this thesis demonstrated that liraglutide promotes anti-inflammatory actions which protect against endothelial and fibroblast dysfunction. Unfortunately, due to time constraints and consequent low numbers of patients, we were unable to specifically interrogate the effects of liraglutide therapy on inflammatory cells profiles and cardiac function in type 2 diabetic patients with comparison to those receiving conventional antidiabetic medication. Nonetheless, the data presented in this chapter appear to highlight differences in inflammatory cell subtypes in type 2 diabetic patients which may be associated with cardiac function, and could therefore be subject to modulation by liraglutide, although this will not be interrogated until full patient recruitment is complete.
6 General discussion

The main aim of this thesis was to investigate the emerging protective effects of the GLP-1 analogue, liraglutide, on cardiac remodelling associated with diabetes, focusing on inflammation as a key regulator of cardiac fibrosis and endothelial dysfunction. The rationale for this project was largely based on our previous finding that a different GLP-1 analogue, exendin-4, attenuated adverse cardiac remodelling in experimental diabetes by specifically altering macrophages infiltration (Tate et al., 2016), and recent clinical data from the LEADER and EXSCEL trials indicating that liraglutide, but not exenatide, confers protection against cardiovascular disease in type 2 diabetes (Hu, 2019).

As part of our first aim, we demonstrated preferential actions of high glucose, with or without liraglutide, to modulate macrophage inflammatory signalling, thereby altering cardiac fibroblast differentiation and endothelial cell dysfunction via paracrine signalling in vitro. NF-κB is considered as a central mediator that regulates proinflammatory signalling involved in different inflammatory and immune processes. Indeed, we demonstrated that high glucose treatment induced NF-κB activation in macrophages, which was not elicited by the same concentration of L-glucose (25mmol/L) as an osmotic control, indicating that hyperglycaemia induces inflammation via post-transcriptional activation of proinflammatory mediators. Furthermore, induction of NF-κB expression in high glucose treated macrophages was associated with functional alterations, specifically increased migration and adhesion, which is implicated in the development of diabetic cardiovascular complications, such as nephropathy and cardiomyopathy (Soetikno et al., 2011). Importantly, NF-κB activation in macrophages was also found to promote secretion of inflammatory cytokines and chemokines in to the surrounding media, which influenced fibroblast differentiation and endothelial cell function in a paracrine manner, via modulation of multiple chemokines and cytokines, including MIP-1α, MIP-1β, and IL-1ra, which are reported to play an essential role in regulating the fibroproliferative process, thereby mediating organ injury and dysfunction (Dobaczewski and Frangogiannis, 2009).

Interestingly, we found that co-treatment with liraglutide reduced high glucose-induced macrophage NF-κB activation, as assessed by protein expression, with consequent modulation of inflammatory genes expression towards a more anti-inflammatory profile (Ma et al., 2018). Consistent with previous reports that cAMP
activation mediates anti-inflammatory effects via inhibition of cytokine and histamine release from mast cells and neutrophils (Moore and Willoughby, 2008) we found that GLP-1R agonists exerted their anti-inflammatory actions through modulation of intracellular cAMP generation. (Figure 3.13) This was specifically assessed by treating macrophages with high glucose with or without liraglutide in the presence or absence of IBMX, as a phosphodiesterase inhibitor which inhibits cAMP metabolism, and/or MDL12330A, as an adenylyl cyclase inhibitor which prevents cAMP formation. In these studies, we found that increased cAMP induced by liraglutide reduced NF-κB nuclear translocation and activation, which was augmented with cAMP activation due to co-treatment with IBMX whilst was abolished with cAMP inhibition due to co-treatment with MDL12330A, in association with induction of cytokine expression (Figure 3.14). As such, our experiments confirmed that NF-κB activity is regulated by cAMP (Minguet et al., 2005), whilst cAMP was specifically induced by liraglutide in high glucose treated macrophages thus altering NF-κB activity. This is consistent with previous studies which have reported inhibitory effects of GLP-1R agonists on NF-κB activation. For example, in a rat model of experimental diabetes, renal NF-κB expression was inhibited by the GLP-1 analogue, exendin-4, which was associated with attenuation of nephropathy (Kodera et al., 2011).

Since alteration of NF-κB expression was observed in high glucose treated macrophages, which was modulated by liraglutide, we also conducted assessment of key functional effects, specifically macrophage migration and adhesion. As previously highlighted, macrophage tissue infiltration and adhesion initiate low-grade inflammation in diabetes by promoting release of inflammatory cytokines and expression of leukocyte adhesion molecules, thereby contributing to characteristic inflammation in, for example, cardiac, endothelial and adipose tissues. Interestingly, we found that liraglutide inhibited high glucose induced macrophage migration, adhesion and altered the expression and secretion of inflammatory mediators, thereby providing further support for our conclusion that liraglutide exerts anti-inflammatory actions which are likely to be at least partly responsible for the apparent beneficial effects against adverse cardiovascular remodelling in diabetes. In this regard, fibrosis and endothelial dysfunction are particular consequences of inflammation in diabetes, whilst macrophages have been identified as key inflammatory precursors (Tate et al., 2016; Tesch, 2010). Specifically, inflammatory cytokines secreted by macrophages in response to high glucose induce fibroblast differentiation characterised by increased expression of α-SMA and CTGF and alteration of ECM proteins, such as MMP-2 and MMP-9, thereby contributing to
characteristic cardiac fibrosis. Notably, it is well established that fibroblasts do not express the GLP-1R, so GLP-1 analogues such as liraglutide are unable to exert direct actions, rather promoting their benefits via modulation of macrophage paracrine signalling, thereby decreasing myofibroblast differentiation expression as an important driver of adverse cardiac remodelling. Notably, conditioned media from high glucose treated macrophages was found to specifically alter expression of the connexins, as key gap junction proteins which play a pivotal role in maintaining electrophysiological conduction between cardiac cells. Indeed, reduced connexin-43 expression has been previously reported in murine ventricular differentiated fibroblasts and ECM remodelling (Zhang et al., 2008), which may lead to conduction slowing and increased incidence of cardiac arrhythmia (Jansen et al., 2012). Consistent with this observation, high glucose conditioned media from liraglutide treated macrophages was found to modulate fibroblast connexin-43 expression, so is likely to support myocardial intercellular conduction, thus improving cardiac structure and function.

Further to our finding that fibroblast differentiation was influenced by conditioned media from high glucose treated macrophages which was modulated by liraglutide, we next interrogated potential underlying signalling pathways known to regulate fibrogenic processes. One likely mechanism by which macrophage paracrine communication may influence fibroblast function is through modulation of Ca$^{2+}$ signalling, which is established to play a pivotal role in pathological remodelling, through for example, TRP channels (Feng et al., 2019). Indeed, our results demonstrated increased expression of TRPV2, which has been previously implicated in regulating Ca$^{2+}$ signalling associated with fibroblast differentiation (Ishii et al., 2018), in fibroblasts treated with high glucose macrophages conditioned media, which was reduced by liraglutide co-treatment. Whilst further mechanistic analysis clear needs to be undertaken, these initial findings suggest that modulation of macrophage paracrine signalling by GLP-1R activation in diabetes may promote alteration of TRP-mediated Ca$^{2+}$ signalling in cardiac fibroblasts, thereby influencing adverse ECM remodelling and progression of diastolic dysfunction.

Endothelial cell dysfunction is known to be a major driver of HF development and progression. It increases peripheral resistance, impairs cardiac coronary perfusion due to reduced endothelium-dependent vasodilation (Zhang, 2008), whilst alteration of barrier function and signalling may specifically drive adverse remodelling thereby worsening ventricular function (Chistiakov et al, 2015). For example, eNOS vasodilator activity is down-regulated whilst endothelin-1 vasoconstrictor activity is
up-regulated, resulting in endothelial dysfunction, vascular remodelling and HF progression (Tabit et al., 2010). As the endothelium is particularly sensitive to and regulates inflammation, the effects of macrophage on cardiac endothelial cells and the influence of liraglutide were investigated. Similar to the fibroblast studies discussed above, cardiac endothelial cells were incubated with high glucose treated macrophage conditioned media with or without liraglutide prior to assessment of gene expression changes. Interestingly, increased expression of endothelial cell adhesion molecules, ICAM-1 and VCAM-1, induced by high glucose macrophage conditioned media, as key markers of endothelial inflammation, was reduced by liraglutide (Figure 3.24). Similarly, expression of endothelial protective factors, sirtuin-1 and eNOS, which was reduced by high glucose macrophage conditioned media, as endogenous anti-inflammatory markers, was restored by liraglutide treatment. Furthermore, hyperglycaemia-induced pro-inflammatory signalling was found to alter expression of endothelial cell junctional proteins, ZO-1, which promote formation of inter-endothelial gaps and vascular leakage/dysfunction, an effect which was also attenuated by liraglutide (Figure 3.26). Taken together, these data, although relatively preliminary, indicate that activation of GLP-1 signalling in macrophages may confer protective actions on neighbouring endothelial cells and fibroblasts, which are likely to at least partly explain apparent protection against adverse cardiac remodelling and dysfunction in diabetes (Schilling et al., 2012; Tate et al., 2016).

Our second aim was to attempt to modify GLP-1 analogues to increase their selectivity for macrophages, thereby potentiating their anti-inflammatory effects. Initially, we relied on the fact that macrophages express mannose receptors (Irache et al., 2008) to add mannose sugar to exendin-4 in order to promote macrophage-specific targeting. We chose attachment of mannose at position 11 (Gallwitz et al., 1994) further to findings of a previous study indicating that this was least likely to alter peptide efficacy. However, assessment of modified exendin-4-mannose efficacy demonstrated significant reduction in exendin-4 receptor binding and activation, thereby necessitating an alternate approach to achieve selective macrophage targeting. Therefore, relying on the characteristic physiological features of macrophages to preferentially take up foreign particles, we decided to exploit nanoparticles as an opportunity for macrophage targeting by designing modified GLP-1 peptides attached to Poly (lactic-co-glycolic acid) (PLGA) nanoparticles. Importantly, attachment to PLGA nanoparticles did not alter liraglutide receptor affinity as determined by GLP-1R internalisation (Figure 4.11) and receptor activity indicated
by cAMP generation (Figure 4.13). In contrast, addition of exendin-4 to PGLA nanoparticles altered receptor binding and activation, possibly due to an inconsistent exendin-4-PLGA formulation (Figure 4.10, 4.12). It should be noted that whilst employment of a nanoparticle-based approach may facilitate targeting of drugs to cells, it has some challenges due to their rapid elimination through macrophage/monocyte phagocytosis (Hu et al., 2019). Indeed, this was confirmed in the present study by live macrophage imaging indicating that liraglutide-PLGA nanoparticles were rapidly taken up by these cells (Figure 4.15). Indeed, it is likely that phagocytosis was so rapid that it occurred before the attached liraglutide had opportunity to bind to the GLP-1R, as this could not be detected by flow cytometry. Indeed, liraglutide-PLGA nanoparticles showed lower affinity for macrophages than liraglutide with respect to GLP-1R activation. In this regard, one potential approach to promote nanoparticle escape from phagocytosis is addition of a polyethylene-glycol (PEG) sheath to cover the nanoparticles and shield them from macrophage phagocytic action (Kelly et al, 2011). It should be noted that in general, PLGA nanoparticles are added to drugs to improve their cell delivery. For example, attachment of GLP-1 peptides to PLGA nanoparticles has been investigated as a novel approach to enhance their oral delivery, since these peptides may only be administered parenterally due to oral instability (Ma et al., 2017). However, native PLGA-modified GLP-1R agonists have not been previously designed for the purposes of selective drug targeting, but have been rather modified by addition of a targeted ligand (Shi et al., 2018).

Finally, we have initiated important clinical studies in order to translate our intriguing experimental findings and to link the evident benefits of liraglutide treatment to the clinical setting. These analyses were specifically designed both to assess whether liraglutide itself may confer direct benefits on cardiac inflammation and remodelling and whether selective targeting of inflammatory pathways may represent a novel therapeutic approach to improve cardiac function in diabetic and non-diabetic HF patients. The large-scale clinical trial, LEADER, established significant reduction in its primary endpoint of cardiovascular death/non-fatal myocardial infarction or non-fatal stroke in liraglutide versus placebo treated patients (Marso et al., 2016b). In order to investigate whether the beneficial cardiovascular effects of liraglutide were linked to its modulatory effects on sub-clinical inflammation, which is known to accompany long-standing diabetes, we interrogated specific effects of liraglutide on circulating inflammatory cell subsets compared to conventional antidiabetic therapy.
in relation to cardiac function. Interestingly, our preliminary findings indicated distinct frequencies of monocytes and T-lymphocytes in diabetic patients with lower proportions of B-lymphocytes and NK-T cells (Figures 5.12, 5.13), whilst macrophage numbers were significantly correlated with early mitral flow velocity (E), which may reflect diastolic dysfunction. In addition, some inflammatory cell populations, such as T-lymphocytes and monocytes, appeared to be positively correlated with other echocardiographic indices, suggesting that circulating inflammatory cell profiles may be indicative of cardiac (dys)function. Unfortunately, however, due to limited patient numbers including in our initial analysis, it was not possible to interrogate the specific influence of liraglutide versus conventional antidiabetic therapy, which will be compared when sufficient numbers of patients in each group have been recruited. In addition to echocardiographic functional analysis and circulating inflammatory cell profiling, future detailed assessment will also include broad analysis of basal and LPS-stimulated cytokine and chemokine expression using comprehensive Luminex assay.

Taken together, the data presented in this thesis provide clear evidence that liraglutide confers anti-inflammatory effects in vitro which indirectly alter fibroblasts differentiation and endothelial cell dysfunction, which are likely to be involved in observed attenuation of adverse cardiac remodelling in diabetes. However, more studies are required to identify specific mechanisms underlying direct cell-cell interaction and the precise anti-fibrotic mechanism of liraglutide. In addition, further development of improved strategies for selective targeting of inflammatory cell GLP-1 signalling is likely to promote such beneficial actions. Finally, as our initial studies have indicated that clinical diabetes may be associated with modulation of inflammatory cell subsets and cardiac function, it is critical that specific actions of liraglutide are investigated in this setting in order to inform more appropriate use of this drug and identify mechanisms underlying beneficial actions on specific inflammatory cells and mediators which may inform novel therapeutic targets for adverse cardiac remodelling and dysfunction in diabetes.
6.1 Study limitations and future work

Complementary to our previous research in laboratory, the data presented in this thesis confirm accumulating evidence of the cardioprotective effects of GLP-1R agonists, particularly liraglutide, in type 2 diabetes, whilst specifically implicating modulation of macrophages inflammatory behaviour and indirect actions on cardiac fibroblasts and endothelial cells. However, there are several limitations that should be discussed and taken in consideration while interpreting the data and for future investigations.

It is important to highlight for some data presented in the thesis, it would be beneficial to increase samples size, particularly for the clinical analyses which had to be performed prematurely due to time constraints. Lack of a normal glucose control group (5.5mmol/L) is an obvious limitation as we only included an L-glucose treated group as an osmotic control for comparison. Whilst, we would argue that L-glucose is an appropriate control, it should be noted that a previous study reported limited effects of the GLP-1 agonist, exendin-4, on an osmotic control group, despite evident effects with normal D-glucose treatment (Younce et al, 2013), suggesting that inclusion of both normal glucose control groups may be important. As much of our in vitro gene expression analysis was limited to mRNA data, it is also clearly essential to extend
this to the protein level, by e.g. immunostaining, which is reflective of posttranslational alterations which are likely to be more consistent with functional changes. An additional limitation of the in vitro macrophage experiments was that end-point assessment of cytokines expression was largely limited to mRNA expression analysis, whilst ELISA quantification of secreted cytokines and chemokines for accurate detection of proteins linked with M1 and/or M2 macrophage polarisation would have been informative in defining specific paracrine signalling mechanisms. For example, one previous study established that high glucose treated macrophages showed increased CD11c and inducible nitric oxide expression under basal conditions (Torres-Castro et al., 2016), whilst other studies have shown that although high glucose treatment alone does not alter basal cytokine expression, addition of LPS as a pro-inflammatory stimulus induced key differences between M1 and M2 cytokine and chemokine secretion (Grosick et al., 2018).

For the conditioned media experiments performed in this thesis, it would be beneficial to treat 3T3 fibroblasts and endothelial cells directly with 25mmol/L LG and/or DG as a control to confirm that the observed alteration of fibroblast profibrotic markers and endothelial cell inflammatory markers was due to exposure to inflammatory cytokines and chemokines specifically secreted by macrophages and not simply due to the high glucose concentration present in the macrophage conditioned media. As well, it would be beneficial to specifically investigate mechanisms underlying paracrine cell signalling and the influence of high glucose/liraglutide in co-culture using both cell contact and non-contact systems, which may help to elucidate the relative impact of physical and chemical cellular interaction.

It is important that future work is undertaken to fill particular gaps in relation to lack of understanding of detailed molecular pathways involved in cardiac fibrosis and/or endothelial dysfunction in diabetes and the specific influence of GLP-1 signalling. The preliminary data included in this thesis suggested that liraglutide may alter changes in fibroblast calcium signalling observed after incubation with high glucose macrophage conditioned media, suggesting that calcium may play a role in cardiac fibrosis and may be a particular target of liraglutide, which is clearly worthy of further investigation. With regard to endothelial cells, we found that cells treated with high glucose macrophage conditioned media demonstrated reduced expression of the anti-inflammatory protein, sirtuin-1, which is a potent inhibitor of NF-κB, which was restored after co-incubation with liraglutide, an interesting finding which also warrants further interrogation. Furthermore, in order to extend the apparently important role of GLP-1R activation in macrophages on suppression of the inflammatory cascade, it
would be interesting to investigate whether the beneficial effects of liraglutide may be accentuated with GLP-1R overexpression, and to interrogate likely GLP-1R independent actions on non-canonical signalling pathways. Finally, to investigate cardiovascular benefits of liraglutide in diabetic patients, it would be preferable to design a double-blind study which was appropriate powered with recruitment of e.g. >200 diabetic patients with specific addition of either liraglutide or placebo to their existing metabolic therapy, with long-term follow-up over a period of 12-24 months. Serial assessment of echocardiographic indices and circulating inflammatory cell profiles and signalling would provide a much more robust analysis of the influence of liraglutide than was possible in the initial pilot study presented in this thesis.
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