Evaluation of metabolic and cardiovascular actions of [Pyr^{1}]apelin-13 in patients with Type 2 diabetes mellitus

by

Petra Sulentic

St Catharine's College

Experimental Medicine and Immunotherapeutics

Research supervisor: Prof. Anthony P. Davenport

June 2022

This dissertation is submitted for the degree of

Doctor of Philosophy

UNIVERSITY OF CAMBRIDGE

Declaration

I declare that this dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text.

The work described here has not been submitted previously for any degree, diploma or other qualification at the University of Cambridge or any other University or similar institution.

The length of this dissertation does not exceed 60,000 words.

Abstract

Name: Dr Petra Sulentic

Thesis title: Evaluation of metabolic and cardiovascular actions of [Pyr^{1}]apelin-13 in patients with Type 2 diabetes mellitus

Type 2 *diabetes mellitus* (T2DM) is common, long term metabolic disorder characterised by hyperglycaemia (high blood glucose) resulting from insulin resistance (IR) and insulin insufficiency. Type 2 *diabetes mellitus* and related complications present a significant cause of morbidity and mortality and there is an urgent need to identify novel pathways that may ultimately lead to the development of new therapies to improve blood glucose control and prevent development of diabetic complications. Apelin is a naturally occurring peptide investigated in animal models and humans showing beneficial cardiovascular and metabolic properties. Apelin reduces peripheral vascular resistance (PVR) and increases cardiac index (CI) in healthy volunteers and heart failure patients, and also increases insulin sensitivity in overweight participants. Besides health risks connected to hyperglycaemia, T2DM patients have increased cardiovascular risk, therefore modulation of the apelin signalling pathway may provide a novel cardiometabolic therapeutic approach. Systemic studies were planned to investigate the effects of prolonged intravenous apelin infusions on CI, blood glucose and IR for the first time in patients with T2DM, following hypothesis that apelin would induce beneficial cardiovascular and metabolic effects in that patient group. Firstly, the tolerability and safety of apelin in increasing doses were tested in a pilot study including healthy volunteers. Subsequently, eighteen participants with increased body mass index (BMI) 25–34.9 kg/m^{2} who served as a model of IR and nine T2DM patients underwent a series of randomised, double blind, saline controlled, prolonged infusion studies. [Pyr^{1}]apelin-13 was infused systemically for two hours with measurements of cardiovascular and metabolic parameters. Results showed that in participants with increased BMI, compared to saline control, [Pyr^{1}]apelin-13 in dose 30 nmol/min caused a significant rise in CI and stroke volume index (SVI), whilst reducing peripheral vascular resistance (PVR) and mean arterial pressure (MAP). After a mixed meal, [Pyr^{1}]apelin-13 also significantly reduced plasma C peptide without inducing significant changes in glucose or insulin plasma level. In patients with T2DM, [Pyr^{1}]apelin-13 in the equivalent [Pyr^{1}]apelin-13 dose of 30 nmol/min and compared to saline control, also increased SVI and CI and reduced PVR without affecting MAP and levels of insulin, glucose or C-peptide. In T2DM group a possible delay in gastric emptying was observed, representing a novel finding in this condition and requiring further investigations. In summary, following these pilot studies results, there is a reason to believe that patients with T2DM have the potential to benefit from apelin mediated vasodilation, increased CI and better glucose homeostasis combined with possible delay in gastric emptying. Therefore, targeting apelin signalling represents novel pathways yet to be explored and developed therapeutically with further studies required to investigate those effects.

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I. Acknowledgements

There are many people I would like to say a special thanks to for their help and support, without whom the work presented in this thesis would not have been possible.

Firstly, I would like to thank Professor Anthony Davenport for supervising me. It has not always been easy, but we made it. Thanks to Professor Ian Wilkinson and Dr Joseph Cheriyan for their guidance and knowledge. There were many fun times which made all the study work more enjoyable. Special thanks go to my AstraZeneca industrial supervisor Dr Philip Ambery, to whom I am very grateful for helping me finding a path towards my next career step.

I would also like to thank Dr Janet Maguire and Dr Carmel McEniery or their advice and technical assistance in the many practical challenges faced during the studies.

I am extremely grateful to my dear colleague and a friend Dr Kaisa Mäki-Petäjä who helped me with her consistent and reliable advice and support throughout and to Mrs Annette Hubsch for her invaluable support, calmness and company during our lengthy studies. I would also like to thank my fellow PhD students Duuamene Nyimanu and Nicola Owen who honestly shared their experiences whilst on a path of their own PhD work.

Many thanks to the nurses in the EMI department Evangelia Vamvaka and Fotini Kaloyirou who often stayed on longer than expected so that the studies would be completed. Very special thanks are extended to my volunteers for their valuable contribution to these studies and to all the kind nursing staff employed at CCRC Unit. In addition, without Mrs Lutchmee Appanah none of this would have been possible, as these studies required lots of time and space which required careful planning. Finally, I am enormously grateful to Dr Fraser Allen and Mrs Jane Kennet for their help in recruiting Type 2 Diabetes patients, which was proven to be a challenging task.

I would like to dedicate this thesis to my loving husband Jon and to my son Borna, who had a little bit less of me, but I will make it up to them. My Mother and Father as always provided their unconditional support and love, especially during these unprecedented Covid-19 times. Thanks to Nina, Branko, Brankica, Jaksa, Gabi and Ana. I never have any doubts in your genuine support.

This study was supported by the British Heart Foundation and AstraZeneca.

II. Publications

Jia, R., Sulentic, P., Xu, J.-M., & Grossman, A. B. (2017). Thymic Neuroendocrine Neoplasms: Biological Behaviour and Therapy. Neuroendocrinology, 105(2), 105–114. https://doi.org/10.1159/000472255

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Read, C., Nyimanu, D., Williams, T. L., Huggins, D. J., Sulentic, P., Macrae, R. G. C., Yang, P., Glen, R. C., Maguire, J. J., & Davenport, A. P. (2019). International Union of Basic and Clinical Pharmacology. CVII. Structure and Pharmacology of the Apelin Receptor with a Recommendation that Elabela/Toddler Is a Second Endogenous Peptide Ligand. Pharmacological Reviews, 71(4), 467–502. https://doi.org/10.1124/pr.119.017533

III. Presentations

Vascular research clinics (VRC) seminar	March 2017
VRC Research day	March 2018
Cambridge MedImmune PhD Programme in Biomedical Research	July 2018
AstraZeneca & University of Cambridge symposium	November 2020

IV. Abbreviations

AT	Adipose Tissue
AE	Adverse Event
α-cells	Alpha Cells
AMPK	AMP-Activated Protein Kinase
ACE2	Angiotensin-Converting Enzyme 2
Ang II	Angiotensin II
APLNR	Apelin Receptor
APLN	Apelin Gene
β-cells	Beta Cells
BP	Blood Pressure
BMI	Body Mass Index
BSA	Body Surface Area
BPS	British Pharmacology Society
CUH	Cambridge University Hospital
CI	Cardiac Index
CO	Cardiac Output
CV	Cardiovascular
CVD	Cardiovascular Disease
CNS	Central Nervous System
CKD	Chronic Kidney Disease
CAD	Coronary Artery Disease
cAMP	Cyclic Adenosine Monophosphate
DNL	De Novo Lypogenesis
DCM	Diabetic Cardiomyopathy
DN	Diabetic Nephropathy
DPN	Diabetic Peripheral Neuropathy

DBP	Diastolic Blood Pressure
ECG	Electrocardiogram
eNOS	Endothelial Nitric Oxide Synthase
ERK1/2	Extracellular Signal-Regulated Kinase 1/2
ET-1	Endothelin 1
FBF	Forearm Blood Flow
FFAs	Free Fatty Acids
FBC	Full Blood Count
GI	Gastrointestinal
GFAP	Glial Fibrillary Acidic Protein
GFB	Glomerular Filtration Barrier
GLP-1	Glucagon-like Peptide 1
GPCR	G-Protein-Coupled Receptor
GCP	Good Clinical Practice
HF	Heart Failure
HR	Heart Rate
ICF	Informed Consent Form
IGT	Impaired Glucose Tolerance
IVGTT	Intravenous Glucose Tolerance Test
IR	Insulin Resistance
KD	Knockdown
LV	Left Ventricle
MAP	Mean Arterial Pressure
mmol/L	Millimoles Per Litre
mins	Minutes
MM	Mixed Meal
MMTT	Mixed Meal Tolerance Test
NO	Nitric Oxide

PDR	Proliferative Diabetic Retinopathy
PIS	Participant Information Sheet
PVR	Peripheral Vascular Resistance
PI3	Phosphoinositide 3-Kinase
POC	Point Of Care
RPE	Retinal Pigment Epithelial
SAE	Serious Adverse Event
SAR	Severe Adverse Reaction
SE	Side Effects
SV	Stroke Volume
SVI	Stroke Volume Index
SF_6	Sulphur Hexafluoride
SBP	Systolic Blood Pressure
TG	Triglycerides
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
VEGF	Vascular Endothelial Growth Factor
VRC	Vascular Research Clinics
V1	Screening Visit
V2–V5	Dosing Visit 2–5
V6	Phone Call Follow Up Visit
WHO	World Health Organisation

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Chapter 1:

Introduction

1.1 Background

Type 2 *diabetes mellitus* (T2DM) is a common, long term metabolic disorder characterised by hyperglycaemia (high blood glucose) resulting from insulin resistance (IR) and relative insulin insufficiency. The risk of developing IR and subsequently T2DM is increased by having excess body weight and also through a sedentary lifestyle. Uncontrolled diabetes can lead to the development of microvascular (diabetic nephropathy, neuropathy, and retinopathy), and macrovascular complications (coronary artery disease, peripheral arterial disease, stroke). As the onset of T2DM can be gradual, physical damage may have occurred prior to diagnosis (Harris *et al.,* 1992; Stolar 2010). While there are many treatments available for T2DM, diabetes related complications may still arise, leading to significant morbidity and mortality (Zheng *et al.,* 2017).

There is therefore an urgent need to identify novel signalling pathways that may contribute to the development of diabetes and related complications. The identification of these pathways may ultimately lead to the development of new therapies targeting better blood glucose control and subsequently preventing these complications.

Blood glucose concentration is closely regulated by the hormones glucagon and insulin secreted in pancreatic alpha (α) and beta (β) cells respectively. After a meal, insulin stimulates glucose uptake in muscle and adipose tissue (AT), as well as glucose storage in the liver (as a form of glycogen). In the fasted state, glucagon stimulates glucose release from stored liver glycogen via glycogenolysis. IR describes the condition at which more than the normal amount of insulin is required for a normal physiological response to glucose increase. In addition to IR being the main underlying disorder in T2DM pathophysiology, it has been independently recognized as a well-established risk factor for cardiovascular (CV) diseases; ranging from atherosclerosis to heart failure, and has therefore been the focus of therapeutic intervention (Patel *et al.,* 2016; Balkau *et al.,* 1999). Under normal metabolic conditions insulin stimulates microvascular perfusion of skeletal muscle and subcutaneous AT and thus increases blood flow, mainly after a meal or physical exercise. That helps not only the delivery of insulin itself, but also of nutrient substrates to multiple tissues beds. This effect is impaired in IR and T2DM (Lambadiari *et al*., 2015).

Historically, insulin was considered the most important peptide hormone in the pathogenesis (development) of diabetes, but today the role of other peptide hormones, such as glucagon-like peptide 1 (GLP-1) and glucagon is becoming well recognised not only in maintaining euglycemia, but also in maintaining blood flow and tissue perfusion (Lim DM *et al.,* 2017; Asmar *et al*., 2017). This highlights the potential of targeting novel pathways in the treatment of T2DM and its associated CV complications. The vascular system controls the delivery of

nutrients and hormones to muscle, and evidence is mounting which links poor blood flow in T2DM and the development of CV disease.

Apelin has been highlighted in animal and human studies as a naturally occurring peptide which inhibits insulin secretion, increases insulin sensitivity, decreases glucose levels and has a role in the pathogenesis of diabetes complications. In addition, apelin increases heart contractility by strengthening the force of heartbeat (positive inotropic actions) and causes widening of blood vessels (act as vasodilator) in the human CV system, which could have beneficial effects in the states of IR and T2DM (Maguire *et al*., 2009; Lambadiari *et al*., 2015).

Therefore, due to the dual beneficial CV and metabolic apelin actions, diabetic patients could benefit from apelin based treatment. The aim was to carry out experimental medicine studies to test this hypothesis in healthy volunteers and in volunteers with increased insulin resistance (overweight/obese and volunteers with T2DM).

This introduction will present the pathogenesis of T2DM and the apelin receptor signalling pathway before summarising the metabolic and cardiovascular apelin effects in preclinical and clinical studies. In addition, the role of apelin in T2DM related complications will be discussed in the context of potential therapeutic targeting of apelin system which could benefit T2DM patients.

1.2 Type 2 diabetes mellitus

T2DM is defined by the World Health Organisation (WHO) as a chronic, metabolic disease characterised by elevated levels of blood sugar (glucose), which over time leads to serious damage to the heart, blood vessels (vasculature), eyes, kidneys and nerves (WHO, 2022).

According to 2019 International Diabetes Federation data, T2DM is a worryingly unrecognised condition as 1 in 2 diabetic patients is undiagnosed. It is known that approximately 463 million adults are already living with diabetes and the number is expected almost to double by the year 2045 (Bhattamisra *et al.,* 2021). The rise in diabetes incidence is highly affected by decrease of socio-economic status and increase in old age. Moreover, lifestyle changes including sedentary habits, high-calorie food and increased life expectancy have led to the global rise in obesity. Obesity, defined as a body mass index (BMI) $\geq$ 30 kg/m^2, is predisposing individuals to IR, which is considered to be major T2DM risk factor and is predating T2DM for approximately 10–15 years (Bellou *et al*., 2018). T2DM patients are having altogether higher percentage of body fat, mostly distributed in the abdominal region (centripetal obesity). T2DM is caused by a combination of IR and relative insulin deficiency. As IR progresses individuals proceed to develop impaired glucose tolerance (IGT) and finally overt hyperglycaemia, clinically diagnosed as *diabetes mellitus* (Solis-Herrera *et al.,* 2021) (Figure 1.1). Glycated

haemoglobin (HbA1c) of 48 mmol/mol (6.5 %) is recommended as the diagnostic diabetes criteria (WHO, 2022).

1.2.1 Insulin and insulin resistance

Insulin is a peptide hormone secreted by pancreatic Langerhans islets β-cells and maintains normal blood glucose (euglycemia) by facilitating cellular glucose uptake of primary target tissues; liver, muscle and AT and regulating carbohydrate, lipid and protein metabolism. In IR normal or elevated insulin level produces attenuated biological response and this classically refers to impaired sensitivity to insulin stimulation (Freeman and Pennings, 2021; Wilcox *et al.*, 2005)

1.2.1.1 Clinical evaluation of insulin resistance

The gold standard IR measurement is hyperinsulinemic-euglycemic glucose clamp technique (DeFronzo *et al.,* 1979). However, this complex technique is primarily used in research and is not in everyday clinical use. Instead, diagnosis of IR is mainly led by clinical presentation and supported by various clinical measurements of IR indexes (HOMA index for example).

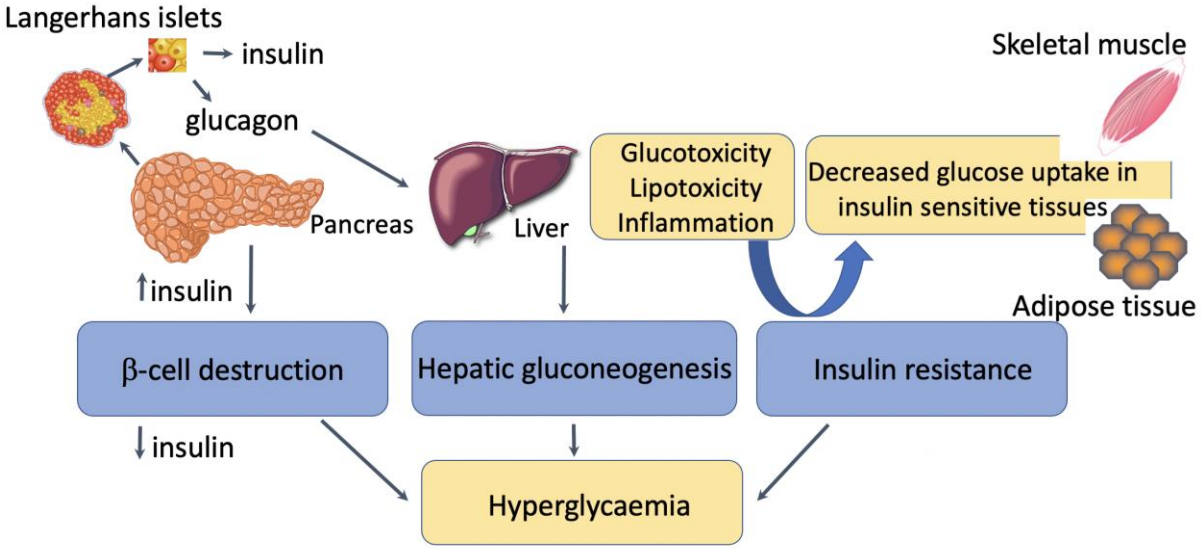
IR can progress to non-alcoholic fatty liver disease, T2DM, metabolic syndrome and cardiovascular disease (CVD) (Freeman and Pennings, 2021).

1.2.1.2 Pathophysiology of IR and T2DM

There are three primary sites of IR; muscle, liver and AT. Muscle is normally accountable for 70 percent of glucose uptake. When calorie load exceeds muscle glucose uptake, glucose is transported to the liver where it triggers *de novo lypogenesis* (DNL) and increases level of circulating triglycerides (TG) and free fatty acids (FFAs), which is causing ectopic fat deposition (adiposity) and inflammation in the muscles, liver and AT. Following fat deposition and inflammation, IR is believed to start in the muscle. IR induced impaired glucose muscle uptake triggers increased delivery of glucose to the liver and DNL, with further increase of circulating FFAs, additionally contributing to adiposity, inflammation and the aggravation of IR. Moreover, failure of insulin due to IR in AT to supress lipolysis additionally increases circulating FFAs levels and muscle tissue steatosis, further contributing to the vicious circle of IR. In the postprandial (after meal) state insulin inhibits liver glycogenolysis and is limiting postprandial glucose rise. However, in the state of IR this mechanism is blunted and hepatic glucose continues to rise. In summary, glucotoxicity resulting from hyperglycaemia alongside lipotoxicity and inflammation contributes to the pathogenesis of IR (Freeman and Pennings, 2021).

Pathophysiology of T2DM is characterised by insufficient insulin secretion due to β-cells dysfunction, tissue IR and increased hepatic gluconeogenesis (Stumwoll *et al.,* 2005; Weyer *et al.,* 1999) as presented in Figure 1.1. In the state of IR there is an attenuated insulin tissue response causing compensatory hyperinsulinemia. In the beginning of IR, β-cells start with a compensatory increase of insulin production in response to the glucose. However, in time that becomes insufficient and is leading to progressive β-cell dysfunction, insulin deficit and relative excess of the hormone glucagon. Glucagon is a peptide hormone stimulating gluconeogenesis, a metabolic pathway that results in the generation of glucose. Gluconeogenesis is additionally contributing to hyperglycaemia, meeting the criteria first for the impaired glucose tolerance (IGT), later followed by T2DM.

Figure 1.1 Pathophysiology of T2DM; triad of β-cell dysfunction, tissue insulin resistance and hepatic gluconeogenesis leading to hyperglycaemia



Glucotoxicity, lipotoxicity and inflammation are causing IR leading to decreased glucose uptake in insulin sensitive tissues (AT and muscle). β-cell is initially compensating IR by increasing β-cell Langerhans islet pancreatic insulin release (↑ insulin). That is gradually causing β-cell loss and absolute insulin deficit (↓ insulin). In addition, β-cell loss is leading to relative excess in hormone glucagon secreted by the α pancreatic Langerhans islets cells and induction of hepatic gluconeogenesis, which is additionally aggravating hyperglycaemia. Hyperglycaemia is caused by the overall decrease in the insulin plasma level due to the β-cell decompensation, decreased glucose uptake in the insulin sensitive tissues due to the IR and to the increase in glucagon stimulating hepatic gluconeogenesis.

1.2.2 T2DM – complications and outcomes

Over time T2DM leads to the development of complications which can be divided into vascular and nonvascular. Microvascular complications are retinopathy, neuropathy, and nephropathy. Macrovascular complications include increased risk of stroke, CVD, and peripheral vascular diseases. T2DM patients have 15 percent increased risk for all-cause mortality compared to people without diabetes, with CVD representing the greatest cause of T2DM associated mortality and morbidity. Meta-analysis confirmed increased risk of coronary heart disease (hazard ratio 2.00: 95 % confidence interval 1.83–2.19), ischaemic stroke (hazard ratio 2.27: 1.95–2.65) and other vascular disease related deaths (hazard ratio 1.73; 1.51–1.98) (Gaede *et al.*, 2003; Emerging Risk Factors Collaboration *et al.*, 2010). In 2019 diabetes caused 4.2 million deaths in a wide range of related complications and co-morbidities which in addition presents great financial burden to global healthcare, highlighting the need for novel treatments. (Bhattamisra *et al.,* 2021).

1.3 Study peptide – [Pyr^{1}]apelin-13

1.3.1 Biology of the apelinergic system

The apelinergic system consists of the Apelin receptor (APJ receptor, APLNR) and the two endogenous peptide ligands; Apelin and Elabela/Toddler. Apelin was considered to be the sole ligand for the activation of the APLNR receptor until the recent discovery of the Elabela/Toddler peptide (Section 1.5), identified by two independent research groups (Chng *et al*., 2013; Pauli *et al.*, 2014).

1.3.1.1 Apelin receptor

Apelin receptor (APJ receptor, APLNR) is a G-protein coupled receptor (GPCR) (Rosenbaum *et al*., 2009) encoded by the APJ gene, localised on chromosome 11 (11q12) and encoding 380 amino-acid protein (O'Dowd *et al.*, 1993). The receptor was identified in 1993 through sequence homology with the gene encoding human angiotensin (Ang) receptor II, type AT1a (O'Dowd *et al.*, 1993). However, despite this homology angiotensin does not bind APLNR to exert physiological effects (De Mota *et al.*, 2000). The receptor remained an orphan receptor until Tatemoto and colleagues in 1998 identified apelin-36 as the APJ ligand; **AP**J **E**ndogenous **Li**ga**n**d (Tatemoto *et al.,* 1998). The APLNR sequence is conserved across species with more than 90 % homology between humans and rodents (O'Dowd *et al.*, 1993; Read *et al.*, 2019).

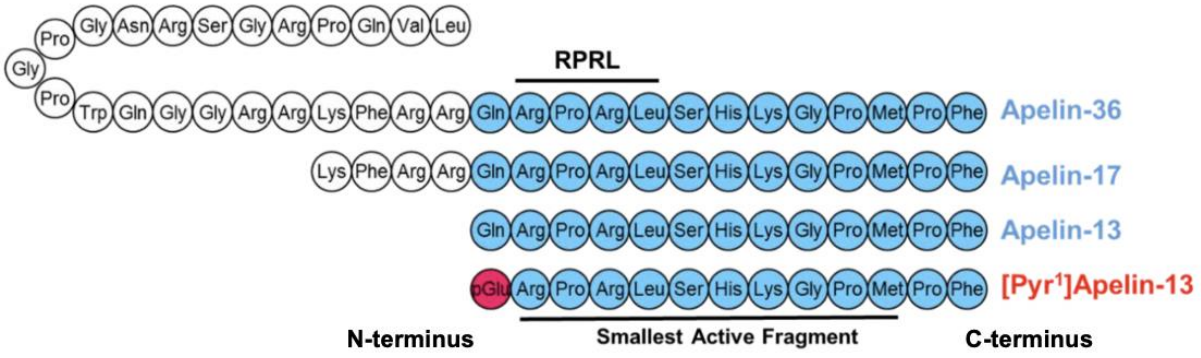
1.3.1.2 Apelin

The human apelin gene (known as APLN) is X chromosome linked; Xq25-26.1 and encodes a 77-amino acid pre-pro-peptide (pre-pro-apelin) (O'Dowd *et al.*, 1993).

Pre-pro-apelin is cleaved to shorter, biologically active isoforms of various lengths including the main apelin fragments; apelin-36, apelin-17, apelin-13 and the [Pyr[1]]apelin-13); pyroglutamate form of apelin-13 (O'Dowd *et al*., 1993; Pitkin *et al.,* 2010; Read *et al.,* 2019). (Figure 1.2).

The origin of apelin in human plasma is not completely clear as the endothelial cells, adipose tissue, heart and skeletal muscle secrete apelin (Kleinz *et al.*, 2005; Boucher *et al.*, 2005.; Ronkainen *et al.*, 2007; Vinel *et al.*, 2018). [Pyr[1]]apelin-13 is the pyroglutamate form of apelin-13 which results from spontaneous cyclization of the N-terminal glutamine, as shown in Figure 1.2. It is the most stable isoform to endopeptidase metabolism and due to higher anti-degradation properties, it has been broadly used for *in vitro* and *in vivo* experiments. Next to apelin-17, it is also the most abundant apelin isoform in the cardiovascular system and human plasma (Pitkin *et al.*, 2010; Zhen *et al*., 2013, Azizi *et al.*, 2008; De Mota *et al.,* 2004; Maguire *et al*., 2009).

Figure 1.2 Apelin



The amino acid sequences of cleaved apelin fragments; Apelin-36; Apelin-17; Apelin-13 and [Pyr[1]]apelin-13 which is shown in red with the pyroglutamate residue in pink. [Pyr[1]]apelin-13 is the most abundant isoform in the human cardiovascular system. Highlighted are RPRL motif critical for the apelin receptor binding and the smallest active fragment [Pyr[1]]apelin-$13_{(2\text{-}11)}$ (Figure from Read *et al.*, 2019 with modifications).

1.3.1.3 Proteolytic apelin peptide degradation

1.3.1.3.1 Endogenous proteases

Apelin peptide metabolism is not fully understood as several metabolic enzymes were shown to be involved in the cleavage of apelin isoforms. To date it is known that angiotensin-converting enzyme 2 (ACE2) (Murza *et al.*, 2014), metalloprotease neprylisin (McKinnie *et al.*, 2016), furin (also known as PCSK3) (Shin *et al.,* 2013) and plasma kallikrein (Fischer *et al*, 2019) cleave apelin peptides. Previously, it was suggested that shorter isoforms result from the proteolysis of longer isoforms with the sequential cleavage of pro-apelin 55 first to apelin-36, followed by apelin-17 and apelin-13 (Kleinz and Davenport, 2005). However, despite those opinions, *in vitro* experiments showed that the enzyme furin can cleave pro-apelin-55 directly into apelin-13 without producing longer isoforms (Shin *et al.*, 2013). ACE2 has been demonstrated to degrade apelin isoforms both *in vitro* and *in vivo*, resulting in the removal of the common C-terminal phenylalanine of apelin-36, apelin-17 and [Pyr^{1}]apelin-13 (Vickers *et al.,* 2002; Wang *et al.,* 2016; Yang *et al*., 2017). It is very interesting that some even shorter fragments retain biological activity. In healthy participants infused with [Pyr^{1}]apelin-13 infusions, the major metabolite generated in the plasma was [Pyr^{1}]apelin-13$_{(1–12)}$, proven to retain biological activity *in vitro and in vivo (*Wang *et al.,* 2016; Yang *et al*., 2017; Nymanu *et al*., 2019). It appears that biological activity is preserved to [Pyr^{1}]apelin-13$_{(1–11)}$, and that the N-terminal glutamine is not essential for receptor binding as [Pyr^{1}]apelin-13$_{(2-11)}$ presents the smallest active fragment, retaining RPRL motif required for binding to the receptor (Zhang *et al*., 2014) For other identified degradation products [Pyr^{1}]apelin-13$_{(1–10)}$ and [Pyr^{1}]apelin-13$_{(1–6)}$ it is not known whether they are biologically active but they retain the RPRL motif enabling binding to the receptor (Nymanu *et al*., 2019). A metalloprotease neprylisin (target for heart failure drug Entresto) fully degrades and inactivates apelin peptide (McKinnie *et al*., 2016), whilst new apelin synthetic analogues with modified neprilysin degradation site (RPRL region) are showing improved proteolytic stability with maintained full cardiovascular actions in mice (McKinnie *et al.,* 2017).

1.3.1.3.2 Apelin half-life

Apelin in both human and rodents has a short half-life of less than 5 minutes (Japp *et al*., 2010; Brame *et al*., 2015), before further degraded by endogenous proteases as was discussed in Section 1.3.1.3.1.

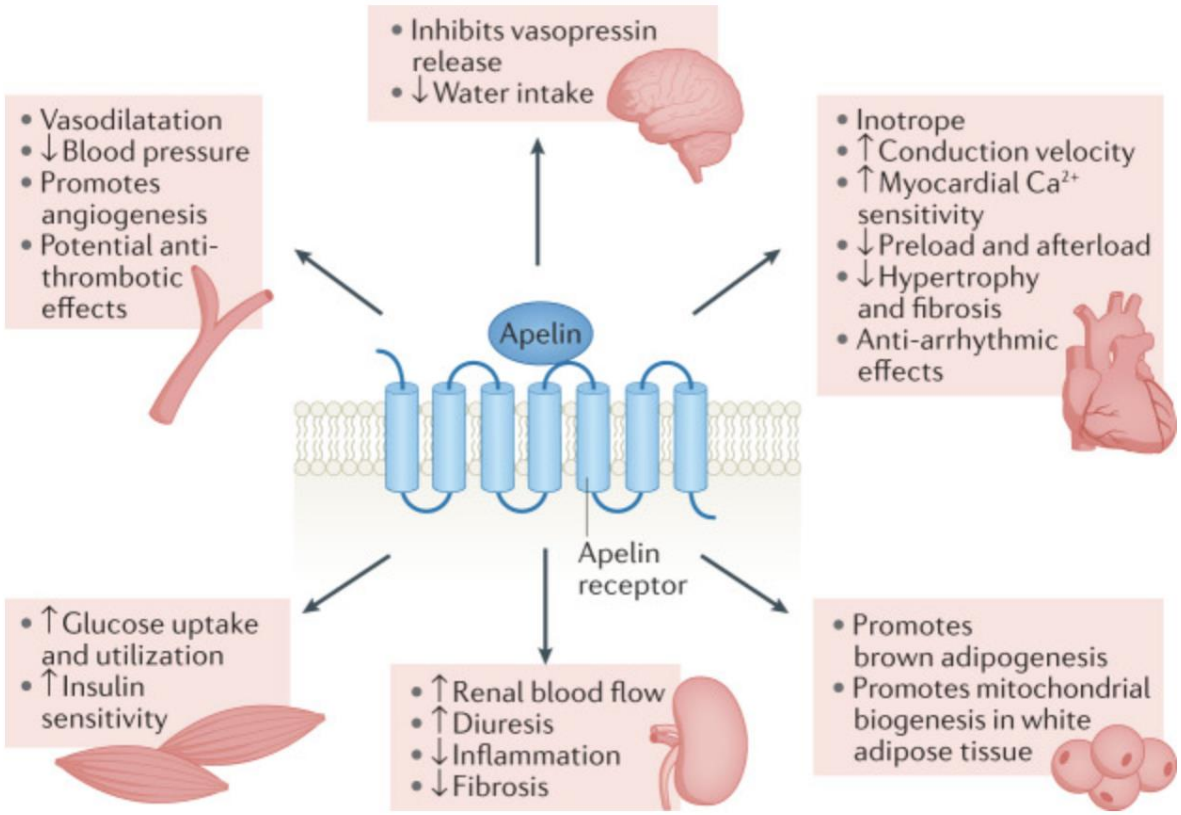
1.3.1.4 Tissue distribution of the apelinergic system

In the human central nervous system (CNS) both apelin and APLNR have been identified in the brain and in the spinal cord (De Mota *et al.*, 2004; De Mota *et al.*, 2000; Lee *et al.*, 2000; O'Carrol *et al*., 2000; O'Carrol *et al.*, 2013). Apelin is highly expressed in breast tissue and in the lungs (Kawamata *et al.*, 2001) and moderately in the heart, liver, gastrointestinal (GI) tract, kidney, gonads and adipose tissue (Edinger *et al.*, 1998; Lee *et al.*, 2000; Kawamata *et al.*, 2001; Medhurst *et al.*, 2003; Kleinz and Davenport 2005). Human APLNR has widespread CNS localisation (O'Carrol *et al.*, 2013). In peripheral tissues, APLNR is similarly distributed to apelin with the highest expression in the spleen and placenta and lower expression in the heart, kidney, lung, GI system and liver (O'Carrol *et al.*, 2000). APLNR and apelin are similarly distributed with apelin having lower serum than tissue concentrations, indicating possible autocrine and paracrine actions (Maguire *et al*., 2009; Chapman *et al.,* 2021).

1.3.1.5 Physiological functions of apelin

The apelin/APJ system is ubiquitously expressed in the cardiovascular and CNS, adipocytes and various other organs playing diverse functions as hormone, adipokine and neurotransmitter (Chapman *et al.*, 2014). The principal physiological functions of apelin isoforms are in fluid homeostasis, modulation of the cardiovascular system and more recently discovered, glucose regulation, which will be the focus of this thesis (Read *et al*., 2019; Chapman *et al*., 2014).

Figure 1.3 Physiological actions of the apelinergic system in various organs



In the cardiovascular system apelin mediates vasodilatation and regulates blood pressure. In the heart, it acts as an inotrope; it is increasing conduction velocity and myocardial sensitivity. Subsequently it is involved in preload and afterload regulation. It promotes angiogenesis and has anti-thrombotic, -fibrotic and -arrhythmic effects. By affecting the hypothalamus and the kidney it is involved in body water regulation; it is inhibiting release of the hormone vasopressin, reducing water intake and increasing renal blood flow and diuresis. It shows kidney protective anti-inflammation and anti-fibrotic properties in preclinical models. In skeletal muscle, it is increasing glucose uptake and insulin sensitivity. Apelin is promoting brown AT adipogenesis and white AT mitochondrial biogenesis. (Figure from Chapman *et al., 2021.)*

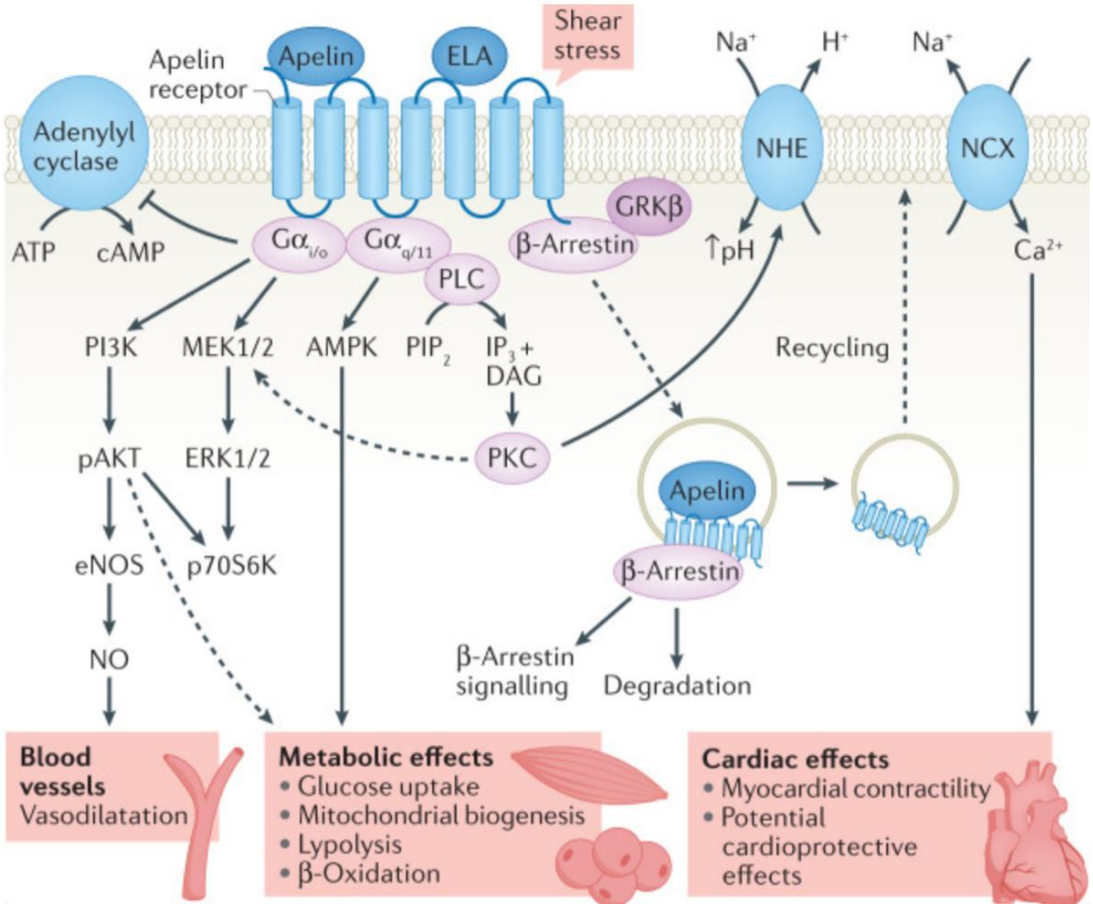
1.3.1.6 Signalling in the apelinergic system

Apelin ligand engages with APLNR and associated G proteins which results in the activation of several signalling canonical cascades. Physiological cardiovascular and metabolic effects vary depending on the apelin isoform and type of activated cell, as is shown in Figure 1.4.

All apelin isoforms (apelin-36, apelin 17, apelin-13 and [Pyr^{1}]apelin-13) block biological effects generated by protein-kinase A (PKA) pathway by coupling Gαi and inhibiting forskolin induced cyclic adenosine monophosphate (cAMP) production. Apelin-12 and -13 stimulate endothelial nitric oxide synthase (eNOS) through activation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signalling pathway, also known as the protein-kinase B (PKB) pathway. Apelin-13 promotes metabolic functions through PI3K/Akt signalling AMP-activated protein kinase (AMPK) pathway. Apelin-13 or -36 activations of the extracellular signal-regulated kinase 1/2 (ERK1/2) is mediated through the protein-kinase C (PKC) pathway by coupling Gαi or Gαq/11. Apelin-13 and [Pyr^{1}]apelin-13 through PI3K/Akt and ERK1/2 activates phosphorylation of ribosomal protein S kinase (p70S6). (Hu *et al*., 2021)

In addition, apelin activates APLNR and mediates receptor desensitisation and endocytosis through β-arrestin-dependant signalling pathway as is shown in Figure 1.4 *In vitro* experiments suggest isoform specific kinetics of apelin/APJ internalisation. Apelin-36/APLNR complex stays localised together with β-arrestin to intracellular lysosome through Rab7 signalling pathway leading to persistent desensitisation of APLNR. In contrast, Apelin-13/APLNR complex is resulting in transient APLNR desensitisation as complex dissociates from β-arrestin rapidly recycling to the surface through the Rab4 signalling pathway (Masri *et al.,* 2006; Lee *et al.*, 2010; Hu *et al*., 2021)

Figure 1.4 Apelin receptor activation is producing metabolic and cardiovascular effects mediated by different signalling canonical pathway



Apelin is leading to vasodilatation in vascular endothelial cells by $G\alpha_i$-mediated inhibition of cAMP production and activation of phosphoinositide 3-kinase (PI3)-AKT signalling cascades (PKB). In cardiomyocytes apelin induces $G\alpha_q$-mediated increase in cardiac (heart related) contractility through activation of the phospholipase C (PLC)-protein kinase C (PKC) pathway with enhanced activity of the Na^+/H^+ exchanger (NCX), further on leading to a rise in intracellular calcium and increased myocardial contractility. The metabolic effects of apelin receptor activation are mediated via PKB or AMPK pathway. APLNR activation recruits β-arrestin required for endocytosis and desensitisation of the APLNR. Apelin/APLNR complex is either lysosome degraded or dissociated from β-arrestin and recycled to the surface. DAG, diacylglycerol; eNOS, endothelial nitric oxide synthase; ERK1, extracellular signal-regulated kinase 1; GRKβ, G protein coupled receptor kinase-β; IP3, inositol tris-phosphate; MEK1, mitogen-activated protein kinase 1; NO, nitric oxide; pAKT, phosphorylated AKT; PIP_2, phosphatidylinositol 4,5-bisphosphonate; p70S6K, p70 ribosomal S6 kinase (Figure from Chapman *et al., 2021.* with modifications*).*

1.3.2 Apelin studies in cardiometabolic disease

A summary of the main preclinical and clinical apelin actions in the cardiovascular system and metabolism are presented in Figure 1.5 and Table 1.1 respectively.

1.3.2.1 Apelin studies in cardiovascular disease

Apelin functions as an endothelium-dependent vasodilator and a potent inotropic agent as summarised in the Sections 1.3.2.1.1 and 1.3.2.1.2. The vasodilation stands for the widening of the blood vessels leading to enhanced blood flow, whilst positive inotropic actions mean an increase in heart contractility.

1.3.2.1.1 Preclinical cardiovascular apelin studies

In vitro human tissues studies conducted by Maguire *et al*. reported apelin peptides to be among the most potent endogenous positive inotropic agents and the endothelium-dependent vasodilators, acting via prostanoid-dependent mechanism. Those experiments additionally revealed direct vasoconstrictor actions in both the artery and the vein upon endothelium removal (Maguire *et al.,* 2009).

In vivo rodent studies showed that acute apelin administration has the nitric-oxide (NO) dependent short-lasting arterial and venous vasodilation effect and is lowering mean arterial pressure (MAP) (Lee *et al.,* 2000; Tatemoto *et al.,* 2001; Cheng *et al*., 2003). Unlike experiments in anesthetised rats (Lee *et al.,* 2000; Tatemoto *et al.,* 2001), experiments in conscious rats (Cheng *et al.,* 2003) showed an increase in heart rate (HR). That effect that can be abolished by ganglion blockade and is suggested to be an indirect reaction due to hypotension (Cheng *et al*., 2003). The discrepancy in the HR response between conscious and non-conscious animals is likely due to the conscious animals having a more active baroreflex system. On chronic apelin administration over the two weeks in rats, the changes in BP and HR did not occur. Nevertheless, the sustained increase in cardiac output (CO) without signs of cardiac hypertrophy has been noted in rats (Ahley *et al.,* 2005). That CO increase is in agreement with the previously conducted experiments confirming apelin inducing positive inotropic effects in normal and failing rat hearts (Szokodi *et al.,* 2002; Berry *et al.,* 2004).

In addition, apelin is reducing cardiac fibrosis, apoptosis, increasing angiogenesis, myocyte glucose uptake and insulin sensitivity, all of which is further explained in Section 1.3.3.1, Table 1.4 and Figure 1.6.

1.3.2.1.2 Clinical cardiovascular apelin studies

The effects of apelin on the cardiovascular system have been investigated in healthy volunteers, patients with central obesity and heart failure (HF), proving apelin to have vasodilatation effects. In 2008 Japp *et al.* investigated for the first time direct vascular effects of [Pyr^{1}]apelin-13 and Apelin-36 in healthy participants showing apelin to be NO-dependant arterial vasodilator without the apparent effect on venous tone (Japp *et al.,* 2008). Further research by Japp *et al.,* has demonstrated that acute apelin administration in healthy volunteers and patients with heart disease causes peripheral and coronary vasodilatation and increases CO (Japp *et al* 2010). Additionally, in 2013 it has been proven that APJ agonism has sustained cardiovascular effects that are preserved in the presence of renin-angiotensin system activation or HF (Barnes *et al.*, 2013). Recently, apelin improved insulin stimulated endothelium-dependant and independent vasodilatation and reduced vasoconstriction mediated by angiotensin II (Ang II) and endothelin 1 (ET-1) (Shinzari *et al*., 2017). Clinical cardiovascular studies are listed in Table 1.1 and will be further addressed in Chapters 3, 4 and 5.

1.3.2.2 Apelin studies in metabolic disease

Apelin inhibits insulin secretion, decreases glucose levels and increases insulin sensitivity, as is explained in the results of the preclinical and clinical studies in Sections 1.3.2.2.1 and 1.3.2.2.2). Increased apelin concentrations in T2DM and obesity are raising the possibility of apelin being a new risk prediction biomarker in the development of T2DM (Noori-Zadeh *et al*., 2019; Ma *et al.,* 2014). In addition, apelin plays a role in the pathogenesis of diabetic complications, which will be addressed separately in Section 1.3.

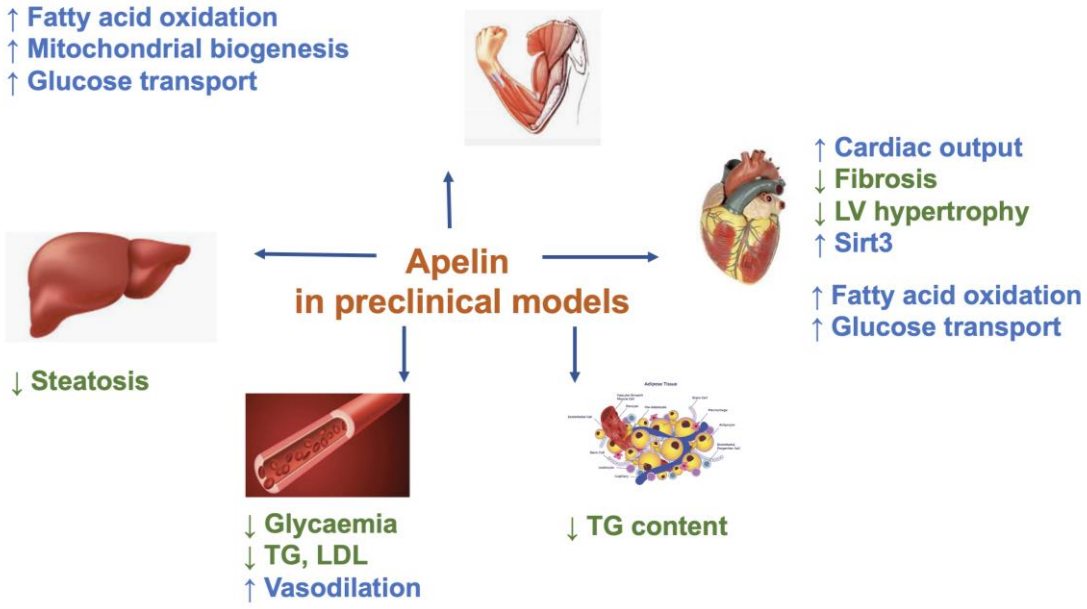
1.3.2.2.1 Preclinical metabolic apelin studies

Both human and murine (rodent) *in vitro* studies showed that apelin is predominantly expressed in α- and β-pancreatic islet cells, while the APLNR is found in human and rodent islets cells and on the INS-1 clonal β-cells (Ringstrom *et al*., 2010). Selective deletion of the APLNR in mice pancreatic islet β-cells resulted in a reduction of islet size, density and β-cell mass. These cells displayed a decreased insulin secretion *in vitro* and the mice showed decreased glucose tolerance (Han *et al*., 2015). Boucher *et al*. demonstrated that in hyperinsulinemia associated obesities insulin exerted direct control on the apelin gene expression in human and rodent adipocytes, leading to plasma and fat cells apelin increase (Boucher *et al*., 2005). In a murine model, apelin-13 injection decreased glycaemia (Dray *et al*., 2008) and both [Pyr^{1}]apelin-13 and apelin-36 inhibited either GLP-1 or glucose stimulated insulin secretion (Sorhede Winzell *et al.,* 2005, Guo *et al*., 2009). Moreover, apelin reduced IR

during a hyperinsulinemic-euglycemic clamp in mice, by stimulating glucose uptake in soleus muscle (Dray *et al*., 2008). The role of apelin in IR has also been studied in apelin deficient mice; these mice were insulin resistant and the resistance was reversed after exogenous [Pyr^{1}]apelin-13 treatment (Yue *et al*., 2010). Furthermore, chronic apelin-13 treatment in mice contributed to improved insulin-stimulated glucose uptake in muscle and to the overall increased insulin sensitivity. Those studies have shown that IR was reduced by increasing fatty acid oxidation and mitochondrial biogenesis in the soleus muscle (Attane *et al*., 2012).

Finally, when apelin was administered in a diabetic rat model, it was found that [Pyr^{1}]apelin-13 could induce a reduction in plasma insulin and glucose levels with a simultaneous reduction in blood pressure (Akcilar *et al.*, 2015). Chronic treatment with acylated analogues of apelin-13 amide ameliorated diet induced obesity and diabetes in mice, together with concurrently improved lipid profile (O'Harte *et al.*, 2018a). Apelin-13 analogues with improved *in vitro* plasma stability, potently lowered glucose and insulin levels *in vitro* (isolated mouse pancreatic islet cells) and in vivo (O'Harte *et al*., 2018b). These studies, therefore, indicate an important role for the apelinergic system in the glucose homeostasis and suggest it could potentially be used as a therapeutic intervention in diabetes patients (Read *et al*., 2019).

Figure 1.5 Summary of the beneficial cardio-metabolic effects of apelin in preclinical research



Apelin is acting as a vasodilator and is improving cardiac performance. It is decreasing both cardiac fibrosis and left ventricular (LV) hypertrophy. Apelin is reducing fat content in adipose tissue, liver and plasma. Apelin is decreasing glycaemia and is improving energy utilisation (Figure from Castan-Laurell *et al*., 2021 with modifications).

1.3.2.2.2 Clinical metabolic apelin studies

Apelin in humans was investigated in the role of a potential therapeutic agent and biomarker in states of impaired glucose homeostasis. A biomarker is a naturally occurring molecule found in the body that is a sign of a normal or abnormal process in disease. Apelin serum concentration was found to be increased in T2DM and obesity based on results of Noori-Zadeh *et al*., metanalyses (2019), raising the possibility of its use as a new risk prediction biomarker in the development of T2DM (Ma *et al.,* 2014).

1.3.2.2.2.1 Interventional metabolic apelin studies

The metabolic effects of intravenously infused apelin in overweight men have been tested in a proof-of-concept study by Gourdy and colleagues who demonstrated that apelin infused over a 2-hour period during hyperinsulinemic euglycemic clamp improves insulin sensitivity (Gourdy *et al.*, 2018). This study is listed in Table 1.1 and will be addressed in more detail in Sections 3.1.1 and 4.7.2. Meanwhile, our research group unpublished data demonstrated a decrease in blood glucose in non-diabetic volunteers after 6-minute apelin intravenous infusion in the dose 100 nmol/min (Brame, 2015; Brame and Davenport, 2015)

Table 1.1 Summary of clinical apelin effects in range of doses in forearm blood flow and systemic studies in distinctive participants groups

Participants	Apelin isoform	Apelin dose	Study type	Apelin action	Reference
Healthy	[Pyr^{1}]apelin-13 Apelin-36	0.1–30 nmol/min	FBF	Vasodilatation in forearm circulation	Japp *et al*.,, 2008
	[Pyr^{1}]apelin-13	1–100 nmol/min	FBF	Vasodilatation in forearm circulation	Brame *et al*., 2015
	[Pyr^{1}]apelin-13$_{(1-12)}$	1–100 nmol/min	FBF	Vasodilatation in forearm circulation	Yang *et al*., 2017
Centrally obese	[Pyr^{1}]apelin-13	1.5 nmol/min	FBF	Increased insulin stimulated vasodilation reduced Ang II and ET-1-dependant vasocontraction	Schinzari *et al*., 2018
Healthy **HF patients**	[Pyr^{1}]apelin-13	30–300 nmol/min	Systemic studies Intracoronary bolus	Peripheral vasodilation Coronary vasodilation Increased CO Reduced cardiac pre- and after-load	Japp *et al*., 2010
Chronic **HF patients**	[Pyr^{1}]apelin-13	30–300 nmol/min	Systemic studies	Peripheral vasodilation Increased CO Improved LV function	Barnes *et al.*, 2013
Healthy overweight	[Pyr^{1}]apelin-13	9 and 30 nmol/kg	Systemic studies	Increased insulin sensitivity	Gourdy *et al.,* 2018

FBF – Forearm Blood Flow; HF – Heart Failure; LV – Left Ventricular; Ang II – Angiotensin II; ET-1 – Endothelin; CO – Cardiac Output

1.3.2.2.2.2 Non-Interventional metabolic apelin studies

Non-interventional clinical studies examined the potential role of apelin as a biomarker in connection to early detection and prediction of T2DM and the evaluation of therapeutic success of diabetes treatment.

1.3.2.2.2.2.1 Apelin as diabetes prediction biomarker in molecular studies

A genetic study carried out in Chinese Hans (T2DM n=1892; healthy controls n=1808) and another smaller study in Iranian women (T2DM n=33; healthy controls n=33) described a connection between some apelin genetic variants, apelin levels and T2DM (Zhang *et al.,* 2009a; Ghafarian-Alipour *et al.,* 2018). However, those discoveries have not been confirmed in people of European descent (n=917) (Sentinelli *et al.,* 2016).

Two prospective studies examined weather apelin has a predictive role in diabetes development (Ma *et al.*, 2014; Castan Laurel *et al.,* 2020) with contradictory results. Ma *et al*., after 3 years follow up found that baseline higher plasma apelin concentrations were associated with higher risk of developing diabetes in Taiwanese non-diabetic men (n=148) (Ma *et al; 2014).* That conclusion has not been confirmed in a more extensive DESIR study, which throughout 9 years follow up on general French population (n=3785) demonstrated that high apelin levels were associated with decreased risk of T2DM development (Castan Laurell *et al.*, 2020).

Serum apelin level among fasting blood glucose, Hba1c and visfatin (all $P<0.05$) was confirmed as part of a subset of significant features for early T2DM detection additionally confirming apelin playing a role in diabetes (Lofti *et al.,* 2020).

1.3.2.2.2.2.2 Observational studies – Apelin serum levels in T2DM and obesity

It was noted by several minute studies that basal plasma apelin levels in obese patients were significantly higher when compared to normal-weight control groups (Boucher *et al*., 2005; Castan-Laurell *et al.,* 2008; Nam *et al*., 2020; Arica *et al.*, 2018). The most likely explanation is that apelin, being an adipokine, is secreted more in obese individuals, due to the higher amount of adipose tissue (Bertrand *et al*., 2015). In agreement to that, female subjects diagnosed with anorexia nervosa (an eating disorder associated with low body weight) had significantly lower apelin levels than healthy controls (Ostrowska *et al.,* 2014).

As obesity is a typical clinical feature of T2DM, then unsurprisingly apelin levels were confirmed to be increased in T2DM patients (Soriguer *et al.,* 2009; Fan *et al*., 2015; Dray *et al*., 2010; Al-Kuraishy *et al*., 2018; Cavallo *et al*., 2012; Yu *et al.*, 2012; Noori-Zadeh *et al*., 2019). Cavallo *et al.* in a large cohort of Italian patients confirmed increased serum apelin only

in T2DM, but not in T1DM patients (Cavallo *et al*., 2012). However, Habchi and colleagues showed apelin to be increased in both T1DM and T2DM with a rise even greater in T1DM (Habchi *et al.*, 2014). Increased serum apelin in T1DM patients was also confirmed by Alexiadou *et al.* (2012). It is known that insulin is upregulating apelin secretion (Boucher *et al*., 2005). Hence, raised apelin levels in T1DM could be due to the exogenous insulin treatment rather than to the endogenous hyperinsulinemia as is the case in T2DM patients.

Apelin serum level stands in negative correlation with HbA1c, and decreases in unregulated diabetes (Habschi *et al.,* 2014). That could explain results coming from Erdem *et al.,* 2008, who showed that apelin serum levels were significantly lower in newly diagnosed and untreated T2DM patients (Erdem *et al.,* 2008). Studies that examined effects of antidiabetic drugs on serum apelin levels found that pharmacologically induced better glycaemic control, independently of the type of treatment was related to the significant increase in serum apelin levels (Kadoglou *et al*., 2010; Al-Kuraishy *et al.,* 2018*;* Fan *et al.*, 2015). Some of the studies investigated in more detail effects of the various antidiabetic drugs. They found that the metformin alone, and even more or in combination with dipeptidyl peptidase-4 (DPP-4) inhibitor increased serum apelin whilst improving glycaemic control and reducing Hba1c (Kadoglou *et al.,* 2010; Fan *et al*., 2015). However, not all studies are in agreement and Yu *et al*., found raised serum apelin in newly discovered T2DM patients, followed by apelin reduction *post* metformin or pioglitazone treatment (Yu *et al.,* 2012).

Recently, Onalan *et al.* studied serum apelin level in subjects with impaired glucose tolerance (IGT), metabolic syndrome and T2DM. They found apelin levels to be significantly lower in all three groups compared to healthy controls, identifying apelin as an important indicator in individuals with IGT in the prediabetic period (Onalan *et al*., 2020).

As apelin is strongly correlating with body fat mass, then unsurprisingly most of the studies testing effects of non-pharmacologic interventions associated with body weight reductions, such as hypocaloric diet, bariatric surgery and exercise showed decrease in serum apelin level in various diabetic and non-diabetic cohorts (Castan Laurell *et al*., 2008; Arica *et al.,* 2018; Soriguer *et al*., 2009; Krist *et al*., 2013). Castan Laurell *et al.* showed weight and fat reduction in 20 obese women was not only accompanied with decreases in serum apelin levels, but also with decrease of mRNA expression of apelin and APLNR in the adipose tissue (Castan Laurell *et al.,* 2008). Krist *et al.,* in a big cross-sectional study (n=740) in obese and T2DM subjects showed that reduction in circulating apelin levels were associated with the reduction in fat mass following exercise, hypocaloric diet or bariatric surgery. That led to significantly improved insulin sensitivity, independently of changes in BMI (Krist *et al*., 2013). As opposed to a majority of studies Hainonen *et al*., did not record significant changes in plasma apelin after diet induced reductions in BMI and suggested that apelin may not be that

strongly correlated with the fat mass despite being an adipokine (Hainonen *et al*., 2009). A series of studies by Kadoglou and colleagues reported every-day moderate physical activity or aerobic exercise up-regulated circulating apelin levels in T2DM patients, even in the absence of significant weight loss (Kadoglou *et al.,* 2012a; Kadoglou *et al*., 2012b; Kadoglou *et al.,* 2013).

Table 1.2 Molecular and observational research studying the role of apelin as a potential biomarker under various clinical circumstances (diabetes, obesity, response to pharmacological and life-style measures)

Study analysing	Author / Year / Study
Apelin genetic variants in connection to T2DM development	Zhang *et al.*, 2009a; Sentinelli *et al.,* 2016; Ghafarian-Alipour *et al.*, 2018
Predictive role of serum apelin in diabetes	Ma *et al.*, 2014, Castan Laurel *et al.,* 2020
Basal plasma apelin levels in relation to the body weight	Boucher *et al.,* 2005; Castan-Laurell *et al*., 2008; Soriguer *et al*., 2009; Ostrowska *et al*., 2014; Arica *et al.*, 2018; Nam *et al*., 2020
Basal plasma apelin levels in diabetes patients	Erdem *et al*., 2008; Dray *et al*., 2010; Yu *et al.*, 2012; Cavallo *et al*., 2012; Alexiadou *et al*., 2012; Habschi *et al*., 2014; Fan *et al*., 2015; Al-Kuraishy *et al*., 2018; Onalan *et al*., 2020; Noori-Zadeh *et al*., 2019 *****
Effects of pharmacologic interventions (antidiabetic drugs) on circulating apelin levels in T2DM patients	Kadoglou *et al*., 2010; Yu *et al*., 2012; Fan *et al*., 2015; Al-Kuraishy *et al*., 2018
Effects of non-pharmacologic interventions on circulating apelin levels in obese participants and T2DM patients	Castan Laurell *et al*., 2008; Hainonen *et al*., 2009; Soriguer *et al*., 2009; Kadoglou *et al*., 2012a; Kadoglou *et al*., 2012b; Kadoglou *et al.*, 2013; Krist *et al*., 2013; Arica *et al*., 2018

***** Meta-analysis

Table 1.3 Observational studies measuring baseline apelin levels in Type 1 and Type 2 diabetes patients

Study / Author / Year	Study population	Study finding
Erdem *et al.*, 2008	T2DM n=40 ND controls n=40	apelin serum levels were significantly lower in newly diagnosed and untreated T2DM patients (*P*<0.001)
Soriguer *et al.*, 2009	MO n=54 (T2DM n=16) ND controls n=12	serum apelin was not raised in the obese subjects unless they were diagnosed with T2DM *P*<0.005
Cavallo *et al.*, 2012	T1D n=131, T2D n=119 ND controls n=137	serum apelin levels were increased only in T2DM, but not in T1DM patients (*P*<0.001)
Alexiadou *et al*., 2012	T1DM n=9 ND controls n=9	apelin concentrations were increased in T1DM patients (*P*<0.001)
Yu *et al.*, 2012	T2DM n=81 ND controls n=79	apelin level was increased (*P*<0.001) in newly discovered T2DM patients
Zhang *et al.*, 2013	T2D n=60 ND controls n=32	serum apelin was significantly higher in T2DM than healthy controls (*P*<0.05)
Habchi *et al.*, 2014	T1DM n=98, T2DM n=162 ND controls n=130	Apelin levels were increased in diabetes (*P*<0.001); with even greater rise in T1DM than in T2DM (*P*<0.02)
Onalan *et al.*, 2020	IGT n=18, MS n=18 T2DM n=18, ND controls n=16	in subjects with IGT, MS and T2DM compared with the ND group serum apelin levels were significantly lower (*P*=0.002, *P*=0.006, and *P*<0.001, respectively)
Noori-Zadeh *et al*., 2019*		Analyses of 16 studies with T2DM subjects (n=1102) and healthy controls (n=1078) presents evidence that apelin circulatory levels are higher in T2DM patients (SMD=2.136 (95 % confidence interval, 1.580–2.693; *P*-value for the significance of the combined SMD examined by the z-test was 0.000)

T2DM – Type 2 Diabetes; T1DM – Type 1 Diabetes; ND controls – Non Diabetic Controls; IGT – Impaired Glucose Tolerance; MS – Metabolic Syndrome; MO – Morbidly Obese; * Meta-analysis

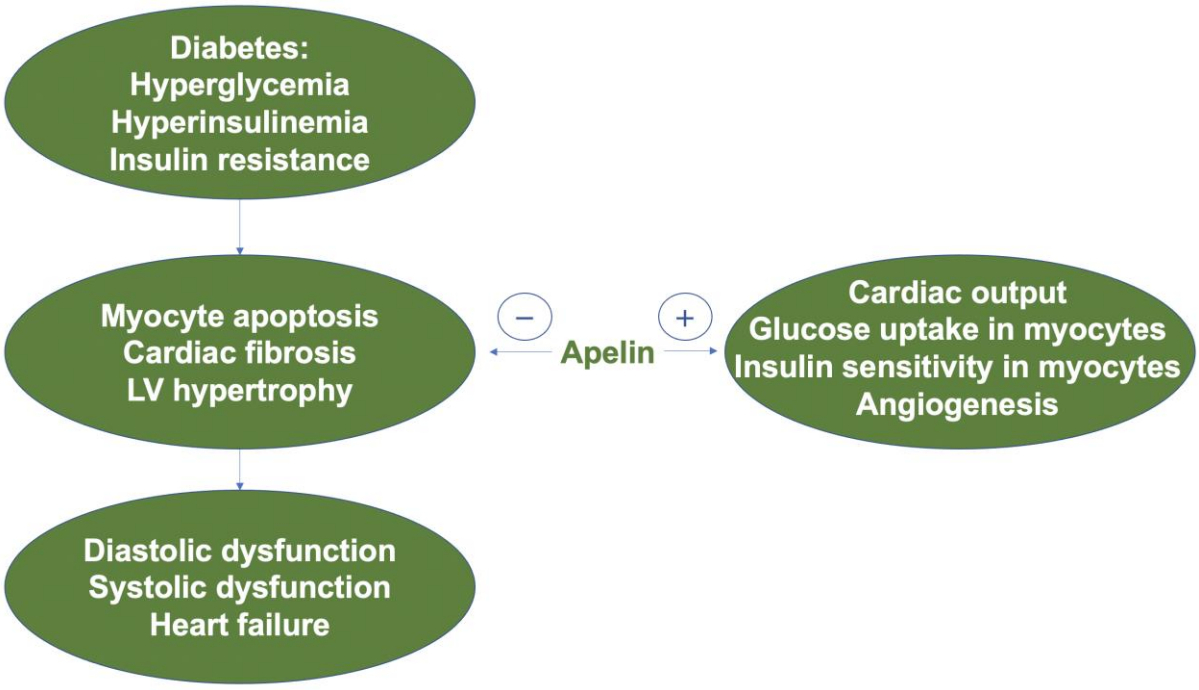
1.3.3 Role of apelin in diabetes complications

Diabetic complications present a major cause of morbidity and mortality in diabetic patients and have previously been listed in Section 1.2.2. Apelin has been recognised to play important roles in the pathogenesis of diabetic complications, reviewed in detail by Hu and colleagues (2016). The majority of the published research has been conducted *in vitro* or in preclinical animal models reporting somewhat contradictory data. Overall, up to date evidence established that apelin may have protective properties in diabetic cardiomyopathy and nephropathy, however with negative impact on diabetic retinopathy as it seems to be promoting retinal neovascularisation. Therefore, before final conclusions weather apelin or apelin antagonists could in the future be therapeutically used for the treatment of diabetic complications, it will be crucial to obtain more human data. Until then, the role of the apelinergic system as a potential treatment of diabetic complications remains unclear.

1.3.3.1 Diabetic cardiomyopathy

Diabetic cardiomyopathy (DCM) is defined as the existence of abnormal heart (cardiac) structure and performance in the absence of other cardiac risk factors, such as coronary artery disease (CAD), hypertension, and significant valvular disease (Jia *et al.,* 2018). It is schematically presented in Figure 1.6 and reviewed in detail in Borghetti *et al.* (2018) and Jia *et al.* (2018).

Figure 1.6 The role of apelin in the pathophysiology of diabetic cardiomyopathy



Hyperglycaemia, hyperinsulinemia and IR start a series of metabolic effects ultimately causing heart cell death (myocyte apoptosis), heart scarring (cardiac fibrosis) and increase in the size of the left heart chamber (left ventricle [LV] hypertrophy). Those changes at first prevent the heart from completely filling with circulating blood (diastolic dysfunction), further progressing to impaired heart chamber contraction (systolic dysfunction) and ultimately leading to heart failure (HF). Apelin is reducing (–) myocyte apoptosis (programmed cell death), cardiac fibrosis and LV hypertrophy. Apelin is increasing (+) cardiac output, myocyte glucose uptake, insulin sensitivity and angiogenesis.

The apelinergic system is identified as a potential therapeutic target in DCM as apelin exerts cardiometabolic effects which can be used in the treatment or prevention of DCM as is presented in Figure 1.6 and Table 1.4.

In vitro and *in vivo* preclinical experiments have proven apelin to be very potent endogenous inotrope agent in both normal and failing hearts with the potential to reduce microvascular insufficiency through vasodilation as previously described in 1.3.2.1.1 (Maguire *et al.,* 2009; Szokodi *et al.,* 2002; Berry *et al.*, 2004; Cheng *et al.,* 2003). Several clinical studies have proven that apelin induces short lasting arterial vasodilation in healthy volunteers (Japp *et al.,* 2008) and increases CO in both healthy volunteers and HF patients (Japp *et al*., 2010; Barnes *et al*., 2013). In addition, apelin increases angiogenesis and vascular density by upregulating the mitochondrial enzyme sirtuin 3, Angs/Tie-2 and VEGF/VEGFR2 expression (Hu *et al*., 2016). Apelin reduces myocyte apoptosis during myocardial injury in rodents subjected to glucose deprivation and ischaemia/reperfusion injury (Zhang *et al.*, 2009b; Boal *et al.,* 2016; Nazari *et al.,* 2020; Li *et al*., 2013; An *et al*., 2020). Apelin protects against cardiac fibrosis and vascular remodelling by blocking Ang II-mediated PAI-1 gene expression in wild type mice (Siddiquee *et al*., 2011), inhibiting TGF-β-stimulated activation of cardiac fibroblasts through a SphK1-dependent mechanism (Pchejetski *et al.,* 2012), supressing TGF-β/Smad2/α-SMA pathway (Lv *et al*., 2020) and inhibiting the PI3K/Akt signalling pathway (Zhong *et al.,* 2020). Apelin is reducing left ventricular hypertrophy (Ashley *et al.,* 2005; Scimia *et al*., 2012; Parikh *et al*., 2018). Metabolically, it increases myocytes FFA oxidation (Ahn *et al.,* 2008), reduces glucose intolerance in obese hyper-insulinemic rodents and increases glucose uptake in the heart (Dray *et al.,* 2008).

Table 1.4 Overview of some preclinical studies demonstrating protective apelin effects in DCM pathophysiology

Apelin effect	Author / Year / Study
Positive inotrope	Szokodi *et al.,* 2002; Berry *et al.*, 2004; Maguire *et al.,* 2009
Vasodilator	Lee *et al.,* 2000; Tatemoto *et al.,* 2001; Cheng *et al.,* 2003; Maguire *et al.,* 2009
Reducing myocite apoptosis	Zhang *et al.,* 2009b; Li *et al.,* 2013; Boal *et al*., 2016; An *et al*., 2020; Nazari *et al.,* 2020
Reducing cardiac fibrosis	Li *et al.,* 2012; Pchejetski *et al.,* 2012; Siddiquee *et al*., 2012; Lv *et al.,* 2020; Zhong *et al.,* 2020
Increasing angiogenesis	Li *et al.,* 2012
Reducing LV hypertrophy	Ashley *et al.,* 2005; Scimia *et al*., 2012; Parikh *et al*., 2018
Increases myocytes glucose uptake and insulin sensitivity	Dray *et al.,* 2008; Xu *et al.,* 2012
Increases myocytes FFA oxidation	Ahn *et al.,* 2008

1.3.3.2 Diabetic retinopathy

Diabetic Retinopathy (DR) is a serious complication of T2DM presenting a worldwide leading cause of vision impairment and blindness with overall prevalence of 22.27 percent (Teo *et al.*, 2021) and the annual incidence ranging between 2.2 and 12.7 percent among T2DM patients (Sabanayagam *et al*., 2019). In clinical medicine, cases of DR with retinal neovascularisation (new blood vessel growth from pre-existing vasculature) are called proliferative DR (PDR) and are distinguished from cases of non-proliferative DR (NPDR), where there is no new blood vessel growth. New retinal blood vessels with abnormal structure are causing leakage into jellylike substance (vitreous humor), which is forming the central part of the eye. Eventually, scarring (fibrotic) tissue from neovascularisation can cause the retinal detachment. In addition, new blood vessels and scarring tissue can obstruct normal flow of the eye fluid and build up pressure (glaucoma) in the eyeball, which can damage the optic nerve and cause blindness (Kinnunen and Yia-Herttuala, 2012)

Apelin may contribute to the PDR by promoting fibrovascular proliferation, as summarised in Table 1.5. *In vitro* experiments reported apelin to be colocalised with angiogenesis endothelial

marker CD31, glial fibrillary acidic protein (GFAP) and vascular endothelial growth factor (VEGF) in diabetic human and rat retinas (Kasai *et al.,* 2004; Liu *et al*., 2010; Qian *et al*. in 2011; Tao *et al.*, 2010; Lu *et al*., 2013). Additionally, fibrovascular role of apelin was confirmed in the experiments with exogenous recombinant apelin, which promoted proliferation, migration and collagen expression in human retinal cells (Qin *et al*., 2013). Moreover, apelin knockdown (KD) experiments and use of the apelin receptor antagonist F13A confirmed apelin involvement in the PDR pathogenesis. Apelin KD by using interfering RNA approach in a model of ischaemic retinopathy suppressed pathological angiogenesis and increased pericyte coverage. Pericytes are cells that provide support to the endothelial cells. Their dropout leads to distorted blood vessel remodelling, potentially causing effects observed clinically as DR (Kasai *et al*, 2013; Beltramo and Porta, 2013). Finally, F13A is reversing retinal gliosis and is suppressing both GFAP and VEGF expression, which may offer new perspectives in the early prevention and treatment of DR (Lu *et al.,* 2013).

In clinical research, apelin was found to be upregulated in the retinal pigment epithelial (RPE) cells and in the vitreous of diabetic patients (Tao *et al*., 2010; Qin *et al.,* 2013) together with vascular endothelial growth factor (VEGF), which is known to have significant role in the pathogenesis of PDR (Kinnunen *et al.,* 2012). However, unlike serum raised VEGF in diabetic patients, data regarding serum apelin levels remain inconclusive, as presented in the Table 1.6 (Tao *et al*., 2010; Du *et al.,* 2014).

In conclusion, it appears that apelin is promoting neovascularisation in DR, which could stand in the way of potential systemic therapeutic use of apelin in diabetes related indications. Due to the retinal adverse effects, more clinical research is needed to illuminate the role of the apelin receptor antagonist, which may become useful agent in the prevention of the retinal neovascularisation.

Table 1.5 Angiogenic and profibrotic apelin effects contributing to the development of proliferative diabetic retinopathy

PDR feature	Apelin effect	Author / Year
angiogenesis	significantly enhanced migration, proliferation, and capillary-like tube formation of retinal endothelial RF/6A cells	Kasai *et al.,* 2004
angiogenesis	promoted VSMC proliferation through PI3K/Akt signalling transduction pathway.	Liu *et al.,* 2010
angiogenesis	Colocalization of apelin and well-established angiogenesis endothelial marker CD31 in PDR patients following vitrectomy	Qian *et al*. 2011 Tao *et al*., 2010.
angiogenesis	apelin knockdown by using interfering RNA approach in a model of ischaemic retinopathy led not only to suppression in pathological angiogenesis but also to increased pericyte coverage	Kasai *et al.,* 2013
pro-fibrotic	recombinant apelin promoted proliferation, migration and collagen I expression in human RPE cells through the PI3/Akt and MEK/Erk signalling	Qin *et al*., 2013
pro-fibrotic angiogenesis	apelin, GFAP and VEGF were significantly increased and co-localised in diabetic rat retinas exogenous apelin promoted retinal glial Müller cells proliferation *in vivo* and induced GFAP and VEGF expression. Apelin receptor antagonist F13A markedly reduced retinal gliosis caused by diabetes and suppressed both GFAP and VEGF expression *in vivo*.	Lu *et al.*, 2013

PDR – Proliferative Diabetic Retinopathy, VSMC – Vascular Smooth Muscle Cells; RPE – Retinal Pigment Epithelial; GFAP – Glial Fibrillary Acidic Protein; VEGF – Vascular Endothelial Growth Factor; RNA – Ribonucleic Acid

Table 1.6 Clinical studies investigating serum levels of apelin and VEGF in T2DM patients with or without DR

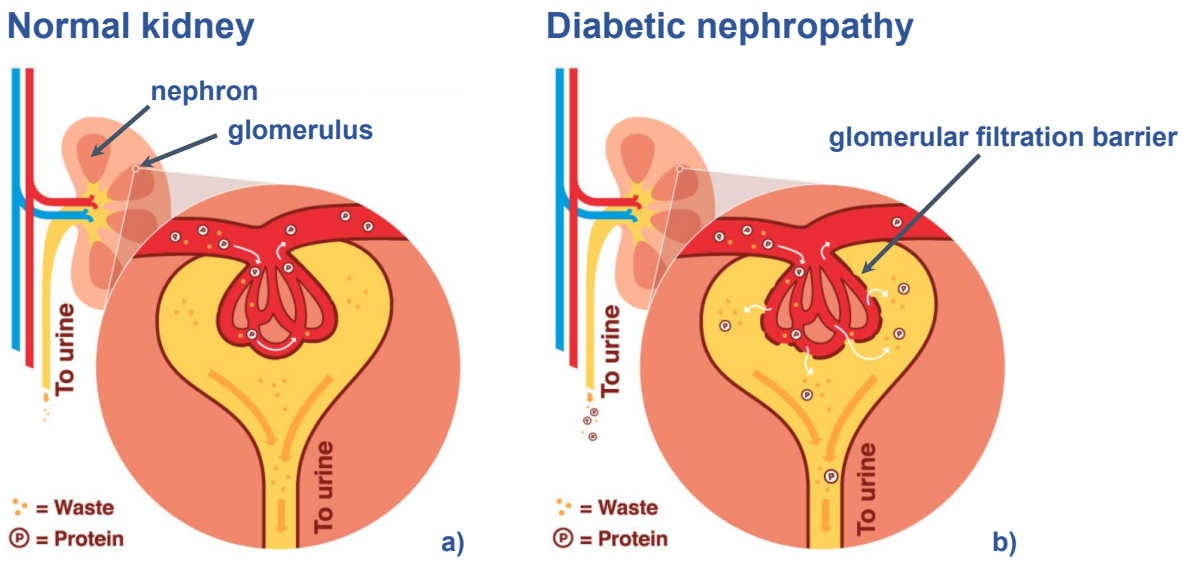
Author / Year	Number (n) of participants	Study finding
Tao *et al.*, 2010	T2DM PDR, n=55 NOD, n=34	vitreous apelin, vitreous and serum VEGF were significantly higher in PDR patients than non-diabetic ocular disease controls (*P*=0.005, *P*<0.001; *P*=0.03 respectively) without significant differences in plasma apelin concentration in-between groups
Du *et al*., 2014	T2DM PDR, n=16 T2DM NPDR, n=23 T2DM non-DR, n=30	Plasma level of apelin-13 was significantly elevated in the PDR group (*P*=0.041), whereas no significant differences of apelin-13 were found between NPDR group and non-DR group. Serum levels of VEGF in the PDR group were found higher compared to the NPDR group and the non-DR group (*P*=0.007, *P*<0.001, respectively). VEGF levels in the NPDR group were found higher than non-DR group (*P*=0.007).

VEGF – Vascular Endothelial Growth Factor; T2DM PDR – T2DM Patients with Proliferative DR; T2DM NPDR – T2DM Patients with Non-Proliferative DR; T2DM non-DR – T2DM Patients without DR; NOD – Non-Diabetic Ocular Diseases Group

1.3.3.3 Diabetic nephropathy

Diabetic nephropathy (DN) presents the most common cause of end-stage renal diseases and is one of the major complications of T2DM. It is estimated 20–40 percent of T2DM patients are affected by DN. Hyperglycaemia changes the way blood flows in the kidney glomeruli (glomerular hemodynamic) and causes an increase in glomerular size (hypertrophy). That leads to changes in the glomerular filtration barrier (GFB) causing an increase in urine albumin (albuminuria). Albumin is a protein found in the blood which normally does not pass GFB (Bruno *et al.,* 2003). Chronic inflammation and pathological neovascularisation also play important parts in diabetes induced renal injury (Donate Correa *et al.,* 2015; Nakagawa *et al.*, 2009). These changes are clinically noted as a gradual decrease in renal (kidney) function (Maezawa *et al*., 2015). Basic kidney structure and changes in the function between normal kidney and DN kidney are shown in Figure 1.7.

Figure 1.7 Changes of the kidney function in diabetic nephropathy



a) Nephron is a functional unit in the kidney, consisted of multiple glomeruli. Glomeruli are producing urine whilst removing waste from blood in the filtration process through the glomerular filtration membrane (GFB)

b) Diabetes is causing changes in kidney function (DN). GFB thickens and becomes more permeable. Proteins (albumins) leak from glomeruli blood vessels and can be detected in the urine (Figure modified from Diabetes UK, 2022).

Studies in human tissues suggested increased expression of the kidney apelinergic system in T2DM patients with DN (Guo *et al*., 2015). It can be hypothesized that apelin, which is increased in the plasma of the T2DM patients plays a role in the development of DN, especially as clinical studies in T2DM patients have proven increased serum apelin to be positively correlating with the albuminuria as is shown in Table 1.7 (Zhang *et al.,* 2013; Guo *et al.,* 2015; Nagib *et al*., 2015). In agreement to those data, experiments in T2DM mice model confirmed apelin induced albuminuria effect, which was later reversed by the use of the apelin antagonist F13A (Zhang *et al.,* 2013).

Another study by Silva *et al.,* suggested positive correlation between serum apelin level and the renal disease progression. However, they interestingly showed a statistically significant decrease in the risk of the hospitalization and CV mortality with higher apelin levels, indicating that apelin might have a clinical use as a marker/predictor of CV mortality and hospitalization (Silva *et al.,* 2013). In contrast, Coskun-Yavuz *et al*., in their clinical evaluation of the relationship between the serum apelin-13 and 1-year progression of chronic kidney disease (CKD) of diabetic and non-diabetic aetiologies have not found correlation between apelin level and a decline in glomerular filtration rate, nor have determined any differences between diabetic and non-diabetic patients (Coskun-Yavuz *et al*., 2015). A summary of the clinical studies examining function of apelin are presented in Table 1.7.

Some preclinical studies are suggesting apelin has a promoting role in DN pathogenesis as is presented in Table 1.8. These experiments suggest apelin induces podocyte dysfunction, increases thickness and permeability of the GFM and promotes pathological glomerular angiogenesis. Some of those effects can be reversed by F13A (Guo *et al.,* 2015; Zhang *et al*., 2013; Liu *et al*., 2017).

However, most of the preclinical studies are mostly advocating apelin is having protective effects on DN by exerting anti-inflammatory and anti-oxidative properties, as is shown in Table 1.9. Oxidative stress and state of chronic inflammation have been shown to play an important part in the DN development (Donate-Correa *et al*., 2015). Day and colleagues observed the significant decrease in the albuminuria and the reduction in glomerular hypertrophy in T1DM Ove26 mice model after long-term administration of apelin-13 (Day *et al*., 2013). Those discoveries were in agreement with Chen *et al.,* who found that apelin reduced proteinuria, glomerular hypertrophy and mesangial expansion (Chen *et al*., 2014). Accompanied with those ameliorative changes in the kidney structure and function, Day and colleagues showed long-term apelin-13 treatment increases anti-oxidant enzyme catalase, which is known to be downregulated in diabetic rat kidney (Day *et. al.*, 2013; Hwang *et al*., 2012). Apelin treatment inhibits diabetes induced renal inflammation in Ove26 and Akita T1DM mice models by inhibiting inflammation pathways and histone hyperacetylation (Chen *et al.,* 2014; Day *et al*.,

2013). In summary, these studies underline that kidney protective apelin properties could be due to the stimulation of anti-oxidative processes and suppression of the chronic inflammation.

In conclusion, most of the available preclinical data are in favour of the protective role of apelin in DN development, whilst studies involving human tissues or diabetic subjects suggest the opposite. Therefore, further clinical investigations should be undertaken to clarify these inconsistencies and to establish the role of apelin in the DN pathogenesis and to investigate therapeutic potential due to the high kidney expression of apelinergic system, clinical importance of DN and elevated serum apelin levels in diabetes (Section 1.3.2.2.2.2.2).

Table 1.7 Clinical research in T2DM patients studying connection between apelinergic system and kidney function

Author / Year	Number (n) of participants	Finding
Coskun-Yavuz *et al*., 2015	T2DM CKD, n=99	no significant correlation between the serum apelin 13 levels and GFR loss (P=0.35).
Zhang *et al*., 2013	T2DM, n=60 NC, n=32	serum apelin was significantly higher in T2DM patients compared to NC group (P<0.05) urinary albumin was positively correlated with serum apelin (R=0.78, P<0.05).
Yonem *et al*., 2009	T2DM non-DR, n=41 T2DM-NPDR, n=23 T2DM-PDR, n=15	positive correlation between apelin levels and urinary albumin/creatinine ratio
Guo *et al.,* 2015	T2DM, n=20 NC, n=20	APLNR expression was increased in kidneys of T2DM patients with DN (P<0.05)

Author / Year	Number (n) of participants	Finding
Silva *et al.,* 2013	T2DM CKD 1 (apelin ≤ 98 pg/mL) n=50 T2DM CKD 2 (apelin 98–328 pg/mL), n=50 T2DM CKD 3 (apelin ≥ 329 pg/mL), n=50	Patients' survival at 83 months in groups 1, 2, and 3 was 39 %, 40 %, and 71.2 %, respectively (*P*=0.046). Apelin levels were negatively correlated with cardiovascular risk factors/CV mortality/hospitalisation and positively correlated with eGFR
Nagib *et al*., 2015	T2DM-NODAT (n=47) T2DM-NDAT (n=40)	Apelin levels in the new-onset diabetes after transplant group (NODAT) were significantly higher than those of the non-diabetic group (NDAT) (*P*<0.001). Serum apelin correlated significantly with fasting blood sugar and proteinuria (*P*<.001, *P*=.001).

CKD – Chronic Kidney Disease; T2DM – Patients With Type 2 Diabetes Mellitus; NC – Normal Control; DR – Diabetic Retinopathy; T2DM-NDR – Patients with No DR; T2DM-NPDR – T2DM Patients with Non-Proliferative DR; T2DM-PDR – T2DM Patients with Proliferative DR; T2DM-1, T2DM-2, T2DM-3 – Three Groups of Patients with T2DM with mild to moderate Diabetic Nephropathy Divided Based On Baseline Apelin Levels; eGFR – Estimated Glomerular Filtration Rate; T2DM-NODAT – New-Onset T2DM Patients after Kidney Transplant; NDCAT – Nondiabetic Control After Transplant

Table 1.8 Preclinical studies in T2DM mice model and cultured podocytes suggesting the role of apelin in promoting diabetic nephropathy and albuminuria

Author / Year	Model	Apelin treatment effect
Guo *et al.,* 2015	KkAy mice cultured podocytes	induces podocyte dysfunction by inhibiting proteasome activities which resulted in ER stress increases thickness of the GFM Effects were reversed by the APLNR antagonist F13A
Zhang *et al*., 2013	KK mice (T2DM model)	increases permeability in glomerular endothelial cells stimulates migration, proliferation, and chemotaxis of glomerular endothelial cells promotes angiogenesis in glomeruli to form abnormal vessels by upregulating the expression of VEGFR2 and Tie2 in glomerular endothelial cells
Liu *et al*., 2017	KkAy mice cultured podocytes	inhibits podocyte autophagy, leading to podocyte apoptosis and renal dysfunction Effects were reversed with APLNR antagonist F13A

Table 1.9 Preclinical studies in T1DM and T2DM mice model suggesting Apelin-13 to be protective of diabetic nephropathy development

Author / Year	Model	Apelin treatment effect
Day *et al.,* 2013	Ove26 mice (T1DM model)	inhibits diabetes induced renal inflammation exerts anti-oxidative properties by upregulating kidney catalase enzyme decreases glomerular hypertrophy
Chen *et al.,* 2014	Akita mice (T1DM model)	inhibits diabetes induced renal inflammation decreases glomerular hypertrophy, mesangial expansion
Muller *et al.,* 2018	db/db mice (T2DM model)	abolishes the proapoptotic effect of high glucose

1.3.3.4 Diabetic neuropathy

Diabetic peripheral neuropathy (DPN) is one of the most common chronic complications of diabetes. DPN prevalence is positively correlated with diabetes duration and severity. While 40 % of patients had DPN by 10 years, 70 % of patients have been affected by 25 years (Knopp *et al.,* 2013). DPN pathogenesis studies focus on hypoxia and metabolic theories, but has not been fully elucidated yet (Bilir *et al.,* 2016).

To date only a limited number of human studies (presented in Table 1.10) addressed the role of apelin in DPN patients. Increased apelin levels were detected in all T2DM patients. However, they were significantly higher in patients with DPN than in patients free of peripheral nerve damage (Bilir *et al.,* 2016; Hosny *et al*., 2019; Xu *et al.,* 2021). Xu *et al*. explored the clinical significance of the serum apelin levels in DPN patients in the context of therapeutic effectiveness. They found that following any treatment (insulin *vs.* insulin combined with nutritional neurotherapy) apelin serum levels reduced, especially in the group treated with nutritional neurotherapy (Xu *et al.,* 2021).

In summary, results of these studies confirm apelin serum levels could potentially be used as a biomarker in T2DM patients with or without DPN. However, further studies with larger sample size and multi-centre design are needed to confirm these results.

Table 1.10 Overview of human studies investigating connection of apelin and diabetic peripheral neuropathy

Author/Year	Number (n) of participants	Study finding
Bilir *et al*., 2016	T2DM, n=99 - DPN, n=46 - non-DPN group, n=53 NC, n=53	Serum apelin levels were significantly higher in all T2DM patients than healthy controls (DPN group and non-DPN *P* values <0.001 and <0.001 respectively). DPN group had higher apelin levels than non-DPN group (*P*<0.001)
Hosny *et al*., 2019	T2DM, n=60 - DPN, n=30 - non-DPN group, n=30 NC, n=20	Serum Apelin levels were significantly increased in DPN group when compared to the control group (*P*<0.001). Highly significant positive correlation between serum Apelin and age, duration of diabetes and BMI in all subjects was noted (*P*< 0.001, 0.001, 0.035 respectively)
Xu *et al*., 2021	T2DM, n=129 - non-DPN, n=44 - DPN – A group, n=41 - DPN – B group, n=44 NC, n=40	Serum levels of apelin were higher in T2DM than those in the NC group, as well as in the DPN group as compared to the non-DPN group (*P* <0.001) Serum levels of apelin in the non-DPN, DPN-A, and DPN-B groups were lower after treatment than before treatment (*P*<0.05). After treatment, serum apelin level in the DPN-B group decreased significantly more (*P*<0.01) and was significantly lower than that of the DPN-A group (*P*<0.05).

T2DM – Type 2 Diabetes Patients; DPN – T2DM Patients with Diabetic Peripheral Neuropathy; non-DPN – T2DM Patients without Diabetic Peripheral Neuropathy; DPN – A – DPN Patients who Received Antihyperglycemic Insulin Treatment; DPN – B – DPN Patients who Received Antihyperglycemic Insulin Treatment Combined with Nutritional Neurotherapy; NC – Normal Control Group.

1.4 Clinical use of apelin agonists and antagonists

There is an emerging consensus that the apelin pathway may be a new important target in cardiometabolic conditions (Castan-Laurell *et al.* 2011, 2012, 2021; Hu *et al.,* 2016). However, the apelin short half-life of under 5 minutes (Japp *et al*., 2010) and the receptor desensitisation (Pope *et al.,* 2016) present a challenge not only in conducting experimental medicine studies, but are also limiting apelin drug development potential. So far, three pharmacological approaches have been identified to overcome pharmacokinetics and pharmacodynamics of endogenous apelin peptide; development of apelin analogues with enhanced biological activity, small molecule apelin agonists and apelin receptor peptidic antagonists. Metabolically stable apelin analogues have been developed through various apelin-modifying approaches with the final effect of significantly increased half-life and enhanced biological activity *in vivo* (for full review please see Huang *et al.,* 2018). Other non-peptidic agonists, small molecules with biased G protein signalling are under development (Read *et al*., 2016). Those molecules do not effectively recruit β-arrestins and are therefore limiting apelin receptor desensitisation and downregulation. Apelin receptor antagonists bind to the apelin receptor and block apelin actions (Castan Laurell *et al.,* 2019; Chapman *et al.,* 2021).

1.5 ELA – second endogenous ligand activating APLNR

Two independent research groups using zebrafish model have identified new peptide Elabela/Toddler to be the second ligand activating APLNR receptor (Chng *et al*., 2013; Pauli *et al.*, 2014). As the research group who reported the discovery first named the peptide Elabela (Chng *et al*., 2013), then the name Elabela (or shortened ELA) has been assigned for future use by the International Union of Pharmacology, Human Genome Organisation and Gene Nomenclature Committee. Name APELA (**AP**elin receptor **E**arly endogenous **L**ig**A**nd) was assigned for the gene encoding this peptide (Read *et al*., 2019).

Chromosome 4 located Apela gene is translated into a 54-amino acid peptide, further degraded by furin enzyme to mature 32 amino acid isoform ELA-32. ELA-32 is further cleaved by furin into ELA-21, ELA-11 (Chng *et al.,* 2013; Pauli *et al.*, 2014) and more recently discovered ELA-22 (Murza *et al.*, 2016). ELA-11 has been observed endogenously in embryos (Pauli *et al.,* 2014). The C-terminal sequence of ELA peptides is well conserved between species, suggesting its critical position for biological function.

Interestingly, despite binding the same APLNR receptor, ELA is less efficacious than apelin in inducing the β-arrestin recruitment, providing a biased functional aspect of ELA *versus* apelin at the receptor signaling level (Zhang *et al.,* 2022). Until recently, ELA half-life was considered to be very short, measuring few minutes (Murza *et al.,* 2016). However, Nymanu et al. (2021)

measured ELA-32 kidney and plasma half-life of 44.2±3 and 47.2±5.7 minutes respectively in mass spectroscopy analyses of plasma and human kidney homogenates (Nyimanu *et al.,* 2021).

ELA is mainly involved in embryonic development, but evidence is growing for Elabela to be involved in physiological and pathophysiological conditions in adulthood (Zheng *et al*., 2021).

APLNR ligands ELA and apelin (described under Section 1.3.1.4) have different tissue distributions. ELA mRNA is downregulated upon embryonic differentiation and development, with highest expression in the blastocysts (Ho *et al.,* 2015). ELA is highly expressed in pluripotent embryonic stem cells and regulates endoderm differentiation and heart development in zebrafish embryos. Upon either ELA or APLNR deletion, similar cardiac embryogenesis defects (lack of hearts) were observed, which suggested ELA to be second APLNR endogenous ligand. These discoveries underlined role of ELA in the embryogenic development of the cardiovascular system (Chng *et al.*, 2013; Pauli *et al.*, 2014). *In vitro* experiments showed ELA-APLNR signalling pathway is functional in the mammalian system where ELA likely serves as a hormone regulating the circulation system in adulthood as well as in embryonic development (Wang *et al*., 2015).

In adults, Elabela has beneficial physiological effects on body fluid homeostasis, cardiovascular health and renal insufficiency, as well as potential benefits for metabolism and diabetes (for full review please address Zhang *et al*., 2018b). The adult tissues ELA has been identified in are human kidney, prostate (Chng *et al.,* 2013; Wang *et al*., 2015), endothelium of arteries and veins, heart and lungs (Yang P. *et al*., 2017b).

In preclinical healthy adult rat model ELA increases cardiac contractility and induces coronary artery vasodilation (Perjés *et al*. 2016; Yang *et al.* 2017b). *Ex vivo*, ELA stimulates angiogenesis and induces relaxation of mouse aortic vessels (*Wang et al.*, 2015). In isolated hearts and in anaesthetised rats, ELA induces positive inotropic effects and decreases MAP (Coquerel *et al.*, 2017; Murza *et al.,* 2016). In pressure-overload mice model of HF, chronic ELA infusion showed attenuatation in myocardial hypertrophy and fibrosis (Sato *et al*., 2017).

In T1DM mice model ELA treatment was observed to induce cardio protective effects and showed SIRT3-mediated Foxo3a deacetylation reduction in oxidative stress, inflammation, fibrosis and apoptosis (Li *et al*., 2021).

Even more than apelin, administration of ELA in rats increases diuresis and thirst without affecting food intake (Deng et al., 2015; Murza et al., 2016). Studies in T1DM mice model have shown that Elabela administration reduces renal inflammation and fibrosis and is leading to renal function improvement by promoting podocyte survival (Zhang *et al.,* 2019).

ELA is secreted at high levels in the adult human healthy kidney, where it is highly expressed in renal collecting ducts and loops. It functions to maintain water and sodium homeostasis and is playing an important role in the pathogenesis of kidney diseases (Zheng *et al*., 2021). To date, only two observational studies evaluated the relationship between serum ELA levels and albuminuria in patients with T2DM and diabetic nephropathy (DN) (Zhang *et al.,* 2018a; Onalan et al., 2020). First Zhang et al. (2018a), showed in 80 T2DM patients that ELA serum levels stand in a negative correlation with urine albuminuria (P<0.001). Further research by Onalan *et al*. (2020), conducted in healthy (n=50) and in T2DM patients (n=100) showed that ELA levels gradually decreases through stages of microalbuminuria and macroalbuminuria in T2DM patients compared to healthy individuals. Those results are suggesting that ELA measurements level should be further investigated, as they could be monitored as clinical biomarker for progression and treatment of DN (Onalan *et al,* 2020).

The effect of ELA in the context of adipose tissue remains unclear and requires further preclinical research (Kolodziejskij *et al.*, 2021).

1.6 Rationale and overarching study hypotheses and aims

1.6.1 Rationale for studies

Following encouraging results in preclinical animal work, there have been a small number of conducted metabolic and cardiovascular *in vivo* human research studies, which have highlighted apelin as a potential novel alternative therapeutic in the conditions of insulin resistance and heart failure (Sections 1.3.2.1.2 and 1.3.2.2.2.1).

Results of those experimental medicine studies suggested that targeting apelin signalling pathways can ultimately achieve the dual outcome of both improving glycaemic control and cardiovascular function. T2DM patients may benefit from improved glucose homeostasis, increased cardiac output and apelin mediated vasodilatation, ultimately leading to enhanced nutrient tissue delivery through improved vascular haemodynamics.

Apelin as a naturally occurring peptide hormone is considered safe for parenteral use due to the short plasma half-life (Section 1.3.1.3.2), without reported side-effects in previously conducted human clinical studies in both healthy volunteers and importantly patients with cardiovascular disease (Sections 1.3.2.1.2 and 1.3.2.2.2.1).

1.6.2 General study hypotheses

Apelin is a potent inotrope and vasodilator in the human cardiovascular system with insulin sensitising properties (Sections 1.3.2.1.2 and 1.3.2.2.2.1) and the apelin receptor represents a potential target for drug intervention in T2DM patients. That will be tested through the following hypotheses:

1. intravenously infused [Pyr^{1}]apelin-13 will be safe and tolerable in increasing doses tested in healthy volunteers
2. intravenously infused [Pyr^{1}]apelin-13 will increase cardiac index (CI) in healthy volunteers, participants with increased BMI and T2DM patients
3. intravenously infused [Pyr^{1}]apelin-13 will increase insulin sensitivity in participants with increased BMI and T2DM patients

These hypothesis will be tested in proof-of-concept studies by systemic infusions of [Pyr^{1}]apelin-13 for two hours to test safety/tolerability and to detect potential cardiovascular and metabolic actions.

1.6.3 General study aims

Aims of the studies were to:

1. test proposed study methods in the pilot studies in healthy volunteers
2. test safety and tolerability of increasing [Pyr^{1}]apelin-13 doses in healthy volunteers, participants with increased BMI and T2DM patients
3. determine [Pyr^{1}]apelin-13 doses likely to produce cardiometabolic effects in participants with increased BMI and T2DM patients
4. investigate the systemic effects of intravenous [Pyr^{1}]apelin-13 on cardiac index, blood glucose levels and insulin sensitivity in healthy volunteers, participants with increased BMI and T2DM patients

Chapter 2:

Methods

2.1 Study design

2.1.1 Ethical and regulatory considerations

An application was made to the local Regional Ethics Committee (REC) for a number of studies (IRAS ID 230996) investigating safety, metabolic and cardiovascular effects of intravenously infused [Pyr^{1}]apelin-13. This research project did not constitute a Clinical Trial of Investigational Medicinal Product (CTIMP) as defined by the EU Directive 2001/20/EC and ethical and regulatory steps have been completed accordingly. The study was registered on Clinicaltrials.gov (NCT03449251) and performed in accordance with declaration of Helsinki and conditions and principles of Good Clinical practice (GCP).

2.1.2 Outline of study design

The project (DEFINE study) was designed as a single-center, saline controlled, randomized experimental medicine study. DEFINE study consisted of three consecutive sub-studies (in further text referred as Study 1, 2 and 3) conducted in three groups of participants; healthy participants, participants with increased BMI (in overweight and class I obese category; see 2.2.2.1) and participants diagnosed with Type 2 diabetes mellitus (T2DM). These studies were completed in full within 12 months and are described in Chapters 3, 4 and 5.

2.1.2.1 DEFINE Study 1 – Dose ranging, open label pilot study to investigate [Pyr^{1}]apelin-13 safety and tolerability in healthy participants

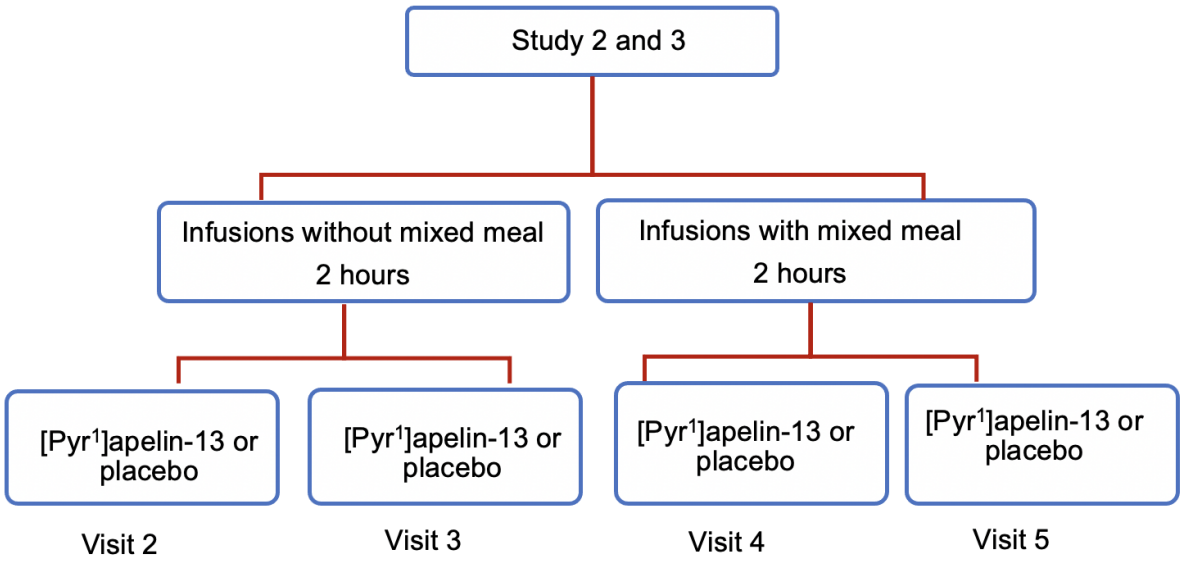
Study 1 was designed to examine safety and tolerability of intravenously infused [Pyr^{1}]apelin-13 in escalating doses in healthy participants, with the aim to define the highest dose threshold for Studies 2 and 3. Study 1 comprised a screening visit (Visit 1), four dosing visits (Visit 2–5) and a phone call follow up visit (Visit 6). On four separate dosing visits each participant was infused with increasing doses of [Pyr^{1}]apelin-13; 30, 90 and 135 nmol/min and normal saline (placebo) as shown in Figure 3.1 (Chapter 3). Selection of tested doses will be explained in Section 3.1.2. Study 1 results were used to decide on the [Pyr^{1}]apelin-13 doses (30, 90, 135 nmol/min) subsequently used in Studies 2 and 3.

2.1.2.2 DEFINE Studies 2 and 3 – Systemic, randomized, double blind studies in participants with increased body mass index (BMI) and Type 2 diabetes mellitus (T2DM)

In Studies 2 and 3 we examined dose dependent cardiac and metabolic actions of intravenously infused [Pyr^{1}]apelin-13 in participants with increased BMI (Study 2) and T2DM patients (Study 3) as is shown in Figure 2.1 and explained in Sections 2.4.2.2 and 2.4.3.5.

Protocol for Studies 2 and 3 will be described in detail in Figures 4.1 and 5.1.

Figure 2.1 Schematic representation of Studies 2 and 3 involving participants with increased BMI and T2DM



All participants attended 4 randomized, double blind visits and received either [Pyr^{1}]apelin-13 or 0.9 % saline control (placebo) on Visit 2 and 3 (for measurement of cardiovascular parameters), followed by [Pyr^{1}]apelin-13 or 0.9 % saline control (placebo) after mixed meal tolerance test (MMTT) on Visit 4 and 5 (for measurement of metabolic parameters).

The results from Study 2 were used to decide which [Pyr^{1}]apelin-13 dose was subsequently used in Study 3. Study 3 followed the same design of visits and randomization as for Study 2.

2.1.3 Methods of blinding and emergency unblinding

Study 1 was a safety study, therefore open-label and both participant and myself (study doctor) were aware whether [Pyr^{1}]apelin-13 or a placebo were infused on the visit.

In Studies 2 and 3 participants and myself (study doctor) were blinded to the infusion prepared by the trained nurse and [Pyr^{1}]apelin-13 and the placebo appeared identical at the point of administration. In the case of an adverse event (AE) that required knowledge of the infusion, it was the responsibility of the principal investigator or myself to unblind the participant and to promptly document and explain the unblinding to the Sponsor and also to provide appropriate management of the participant.

2.1.4 Study stopping criteria

An individual visit was to be terminated early if the participant wished to do so for any reason. Also, I as the study doctor, could terminate the visit if there was evidence of significant clinical deterioration. The DEFINE study was to be terminated early in the event of a severe serious adverse effect (SAR) linked to the [Pyr^{1}]apelin-13.

2.2. Study participants

2.2.1 Participants recruitment

Participants were recruited on an outpatient basis and were identified by their treating clinicians in clinics, or from existing research databases where available. Advertisements were also used and placed in hospital waiting rooms, in public areas within the Cambridge University Hospital (CUH), Cambridge University and local community, nearby GP surgeries, radio, internal and external websites, and distributed via email. The study was also promoted through word of mouth.

2.2.2 Inclusion, exclusion criteria and participants study restrictions

2.2.2.1 Inclusion Criteria

Studies were conducted in three groups of participants; healthy (Study 1), overweight/class I obese (Study 2) and in those diagnosed with T2DM (Study 3).

The selection of study participants was defined according to the BMI, waist diameter, percentage of total body fat measurements and relevant medical history, all of which will be described in Sections 3.4, 4.4 and 5.4. All participants were current non-smokers, aged 18 to

70 years, male or female. Those female participants of child-bearing potential were asked to use adequate contraception and checked to have negative pregnancy test performed at the beginning of each visit.

Study 1 participants were required to have a BMI in range 18.0–27 kg/m^2 with either waist circumference lower than 88 centimetres (35 inches) for women and 102 cm (40 inches) for men, and/or body fat percentage less than 32 % for women and 25 % for men. Participants in Study 2 had increased BMI to match WHO criteria for overweight (BMI 25–29.9 kg/m^2) and within the class 1 obesity category (BMI 30–34.9 kg/m^2), but were otherwise healthy. The BMI was within the range of 25–34.9 kg/m^2 with either waist circumference higher than 88cm (35 inches) for women or 102 cm (40 inches) for men, and/or body fat levels in excess of 32 % for women and 25 % for men. Participants were invited to take part in Study 3 only if they had documented diagnosis of T2DM and were either diet controlled or treated with oral antihyperglycemic therapy, with BMI measured 18–34.9 kg/m^2.

2.2.2.2 Exclusion Criteria

The absence of significant abnormalities in clinical examination and blood results was systematically required in addition to normal baseline cardiovascular parameters.

Unless documented with T2DM in Study 3, all participants were free of known chronic diseases relevant to the studies.

2.2.3 Participant study duration

Participants were required to attend one screening visit (Visit 1 – V1), four dosing visits (Visit 2 – Visit 5) and to be contacted over telephone for follow up visit (Visit 6). Screening and dosing visits were organized at mutual convenience of the participant and study team in accordance with the availability of study facilities, either in consecutive days or up to 28 days after the previous visit. A final follow-up phone call (Visit 6) was made to the participants 24–48 hours after their last dosing visit to assess AEs. Each screening visit lasted 1 hour and dosing visit up to 4 hours.

2.2.4 Participant withdrawal criteria and completion of the study

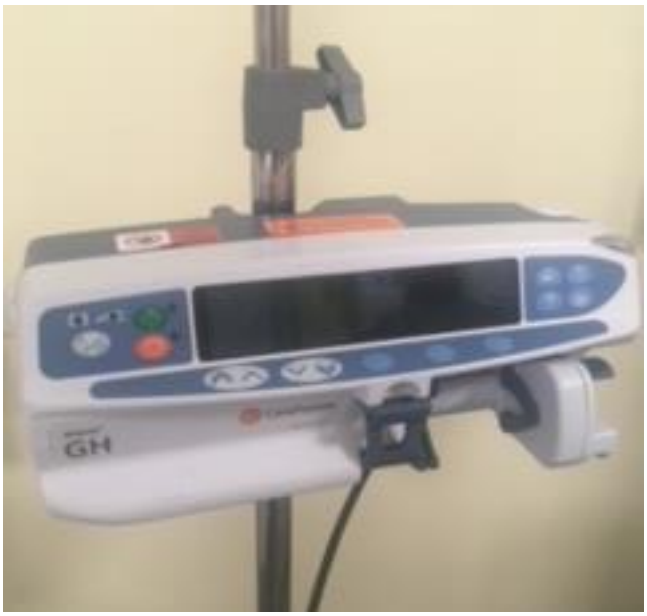
It was within the remit of the study doctor to withdraw a participant for appropriate medical reasons, individual AEs or if new information was gained about the compound of interest or study related procedures. Study participants that had been withdrawn from the study received a follow-up telephone call within 24–48 hours of their last attendance. Participants were considered completed if they finished all study visits, including the follow-up visit.

2.3 Study treatment

2.3.1 Route of study treatment administration

[Pyr[1]]apelin-13 or a placebo infusion was administered for two hours by means of venous cannula inserted in the antecubital or hand veins of either right or left arms, at a steady infusion rate 1 ml/min (Alaris GH syringe pump).

Figure 2.2 Alaris GH syringe pump was used in all studies for intravenous infusion of [Pyr[1]]apelin-13 peptide or placebo



2.3.2 [Pyr[1]]apelin-13

[Pyr[1]]apelin-13 used for clinical studies was custom synthesized by Severn Biotech Ltd. (Kidderminster, UK).

Peptides were synthesized to Good Manufacturing Practice (GMP) standard by Severn Biotech, Kidderminster, UK using Fmoc chemistry on a solid phase support matrix. They were over >95 % pure (confirmed with mass spectroscopy and reverse phase high pressure liquid chromatography; RP-HPLC), tested and confirmed to be sterile and pyrogen free by Wickham Laboratories Ltd., Gosport, UK. The peptides were supplied in sealed glass vials as white powder and stored at -40 °C. Each batch was tested to confirm biological activity by constructing a concentration response curve and EC_{50} values calculated (Eurofins DiscoverX) in β-arrestin assay. On the study day, [Pyr[1]]apelin-13 was allowed to warm to room temperature and was diluted as per protocol with normal saline to produce stock solutions,

which were then filtered using a 0.2 micron Portex flat filter (Portex, UK) before undergoing serial dilutions with normal saline to prepare infusion. Dilutions and infusion preparation were conducted by a delegated member of the study team (nurse) in an open label (Study 1) or double-blind randomized manner (Study 2 and Study 3) in accordance with the study protocol (see 2.1.2 and 2.1.3). Once prepared, [Pyr^{1}]apelin-13 infusion was stable at the room temperature.

2.3.3 Normal saline

On visits in which [Pyr^{1}]apelin-13 was not scheduled, participants received normal saline as a non-active placebo treatment.

2.4 Study procedures and assessments

All study related procedures and assessments were carried out by clinically and GCP trained staff on clinical research units; Addenbrooke's Centre for Clinical Investigation and Cambridge Clinical Research Centre of CUH.

2.4.1 Participant identification, information sheet and consent form

Prior to any study specific activity was performed, it was ensured that each participant had access to a Participant Information Sheet (PIS) for at least 24 hours and to read it carefully before signing the Informed Consent form (ICF). Both the ICF and PIS were in compliance with regulatory and legal requirements, approved by the REC and they ensured each participant was fully informed about the nature of peptide, objectives of the study and possible risks associated with their participation. After consenting, participants were allocated a unique participant identification number (Participant ID), which was used for their identification throughout the study. The voluntary nature of participation and the ability to withdraw an individual's consent at any time was reiterated at each visit and participants were provided with a contact number on the PIS to be enabled to ask any questions or to get in touch at any time. Participants communicated with the research team at study visits, over the telephone or mobile, e-mail or in writing. Communication with participants was clearly documented in the participant's notes.

2.4.2 Schedule of assessments

Summary of screening and dosing visit assessments is shown in Figure 2.3.

2.4.2.1 Screening visit assessments

The screening visit (Visit 1) included medical history review, physical examination, anthropometric measurements, blood analyses and ECG to determine eligibility.

Randomization in Studies 2 and 3 took place once eligibility was confirmed, prior to the first dosing visit.

2.4.2.2 Dosing visits assessments

Participants attended four dosing visits (Visit 2–5) on separate days in duration of 4 hours per visit, to receive intravenous infusions of either [Pyr^{1}]apelin-13 or a placebo, as shown in Figures 2.1 and 3.1. All study visits were organized in a quiet, temperature controlled (23–25 °C) room for the duration of the study. Twenty-four hours prior to dosing visits participants were asked not to take any anti-inflammatory medications, alcohol or caffeine. On the day of the study, they could have attended fasted or had a light breakfast at least 4 hours before the visit. Cardiovascular parameters (visits 2 and 3) and metabolic parameters (visit 4 and 5) in an individual, were measured at the same time of the day to reduce variability. Participants were instructed to withhold their regular medications on the study day.

Before the infusion was started, each participant's medical history, concomitant medications, and AEs were reviewed. After the participant was lying down and feeling comfortable in a supine position, the study doctor performed brief physical examination followed by 12 lead ECG recording and cannulation of both arms to establish venous access for blood sampling and infusion. Two venous cannulas (18–22 gauge) were inserted into bilateral large antecubital or hand veins for peptide infusion and sampling of venous blood in the opposite arm to the infusion site.

During a two-hour long infusion, venous blood samples, vital signs, point of care (POC) capillary glucose and cardiovascular hemodynamic measurements were taken at predefined times whilst cardiac telemetry recording was continuous. Details of the predefined time points for Studies 1, 2 and 3 dosing assessments are shown in Figures 3.2 and 4.2. Some of the visits in Studies 2 and 3 included MMTT, as was shown in Figure 2.1 and explained in Section 2.4.3.5.

Post infusion participants had their 12 lead ECG recorded before being offered lunch and regular medicines, if not taken on the study morning.

Figure 2.3 Summary of study screening and dosing visit assessments

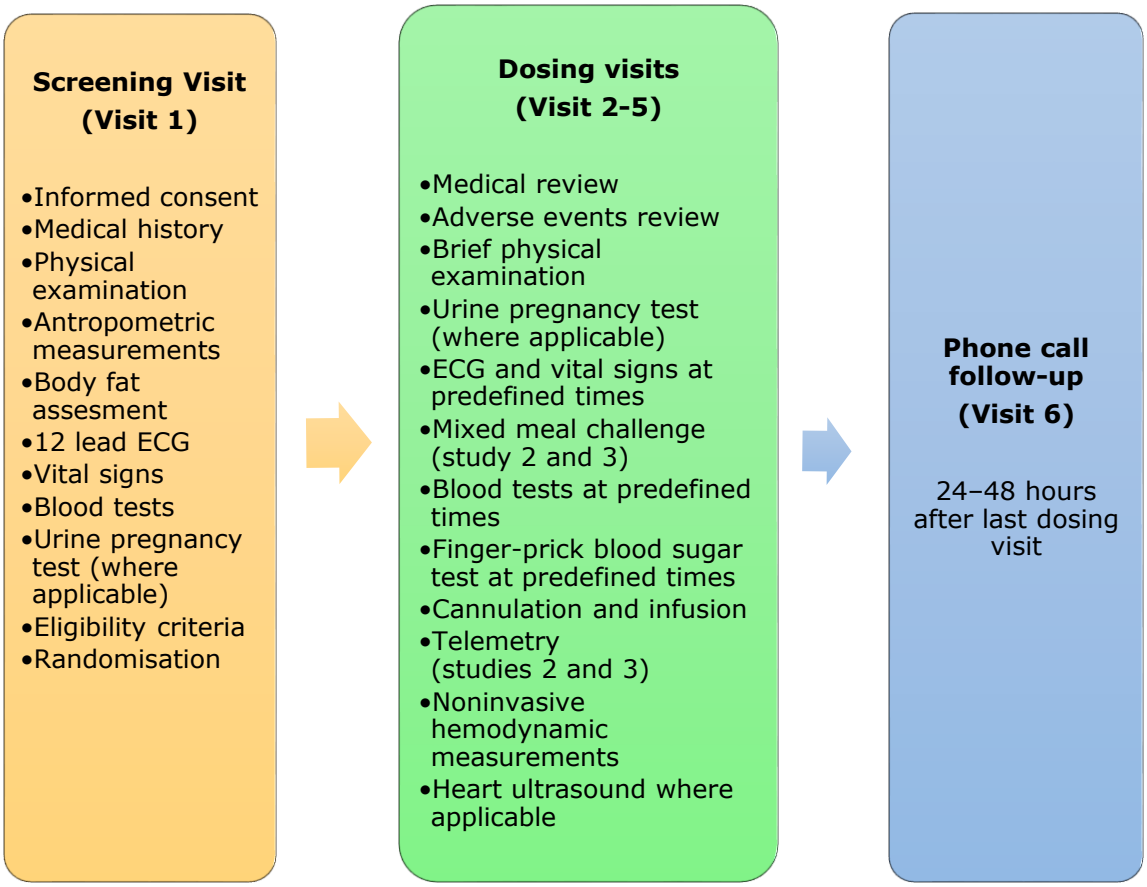


Figure 2.4 Demonstration of the study setting.

With permission of Dr James Goodman and Mrs Annette Hubsch (in the Figure)

2.4.3 Description of study related procedures and assessments

2.4.3.1 Anthropometric measurements

Each participant's weight, height, waist circumference and percentage of body fat was assessed on the screening visit (Visit 1) as part of the eligibility criteria assessment.

Body fat percentage and weight were measured by body composition weight scale (Tanita MC-780MA P; Amsterdam, Netherlands), whilst height was measured by standard tape and expressed in SI units.

The Tanita scale utilises bioelectrical impedance analysis to measure body composition. The scale sends an imperceptible electrical current through the body. Muscle, fat, bone and water conduct electricity at different resistance rates, which are then measured by the scale. The body composition scale processes this information combined with data such as gender, age and height. After calculations are made very quickly, it can tell a lot of details about the composition of the body as body fat percentage as well as BMI calculated as weight in kilograms divided by square of height in meters; BMI=kg/m^2.

Figure 2.5 Tanita scale

Participant is holding the handles and standing on Tanita scale. Following data input (gender and height) the scale calculates body weight, body fat percentage and BMI.

2.4.3.2 Venous samples for blood analysis

Venous blood samples were collected by venepuncture or through venous cannula per standard protocol before being processed at Clinical Biochemistry and Core Biochemical Assay Laboratory of CUH.

On screening visits (Visit 1), samples for full blood count (FBC), renal and liver function and glycosylated hemoglobin (Hba1c) were analysed as part of the inclusion criteria.

On dosing visits (Visits 2–5) participant blood samples were taken throughout the study at predefined times during the infusion (at baseline, 15, 30, 45, 60, 75, 90, 105 minutes and at the end of the infusion) (Figure 3.2 and 4.2). Blood samples for insulin, C peptide and apelin were drawn into lithium heparin tubes and kept on ice before centrifugation (7 minutes at 4–8 °C, 4000 revolutions per minute). Platelet-free plasma was decanted and stored at -70 °C before measurements of insulin, C peptide and apelin were made. Insulin and C peptide were measured by sandwich chemiluminescence immunoassay (DiaSorin Ltd, Saluggia, Italy), and apelin by competitive enzyme immunoassay (EIA) RedDot Biotech Human Apelin assay (Stratech Ltd). Glucose samples were drawn into serum separating tubes. Samples processed and analysed on the day of the study were FBC and glucose.

2.4.3.3 Point of care (POC) capillary blood glucose monitoring

Tests were performed for additional safety blood sugar (glucose) testing throughout dosing visit infusions at predefined times. The condition where blood glucose is lower than normal is called hypoglycaemia, defined usually at blood glucose level of less than 3.9 millimoles per litre (mmol/L). Hyperglycaemia, the state of blood glucose being higher than normal is usually defined as higher than 7mmol/L before eating and 8.5–9 mmol/L two hours after a meal. Samples were taken by standard finger prick testing and analysed with a standard blood glucose meter (Accu-Check glucose monitor, Roche, France).

2.4.3.4 Urine pregnancy test

Testing for pregnancy was performed in female participants if participant was of child bearing age (Invitech, Guangzhou Wondfo Biotech CO., Ltd) on the screening visit and before every dosing visit.

2.4.3.5 Mixed Meal Tolerance Test (MMTT)

A Mixed Meal Tolerance Test (MMTT) was used in Studies 2 and 3 as a dietary challenge (mixed meal [MM] challenge) to compare changes in postprandial secretion of glucose, insulin and C peptide in two treatment groups ([Pyr^{1}]apelin-13 or a placebo) on Visit 4 and 5. Volunteers were provided with a standardized 125ml shake drink (Fortisip Compact vanilla flavor; Nutricia Ltd.) which contains a mixture of 37.1 g carbohydrate, 11.6 g fat and 12 g of protein. The drink was consumed within a 5-minute period and blood samples for glucose, insulin and C peptide were collected at baseline before the drink was consumed and at predefined time intervals throughout infusion. The meal induces a raise in blood glucose, which leads to pancreatic beta cells insulin release, which subsequently causes a raise in the insulin degradation product C peptide. Analyses were made to detect postprandial changes in metabolic parameters and to obtain conclusions of changes in insulin sensitivity.

Figure 2.6 Fortisip compact used as a mixed meal challenge in Studies 2 and 3

2.4.3.6 Infusion

[Pyr^{1}]apelin-13 or normal saline (placebo) were administered as described under 2.3.1 and 2.1.3

2.4.3.7 Cardiovascular measurements and recordings

2.4.3.7.1 ECG measurements

Standard 12-lead electrocardiogram (ECG) recordings were taken at the screening visit, before the start and at the end of each dosing visit.

2.4.3.7.2 Cardiac telemetry

Cardiac telemetry is a portable device that continually monitors patient ECG while automatically transmitting information to a central monitor. Continuous ECG recording was performed throughout the whole 120-minute infusion period and reviewed after each dosing visit completion to ensure safety.

2.4.3.7.3 Vital signs (blood pressure and heart rate)

Blood pressure and heart rate were measured using a semi-automated oscillometric sphygmomanometer (Omron 705IT, Japan) shown below on Figure 2.7 at pre-defined times during the study. Measurements of systolic and diastolic blood pressure (SBP and DBP respectively) were used to calculate mean arterial pressure (MAP). MAP is the average pressure in the arteries during one cardiac cycle, with normal range 70–105 mmHg. It was calculated as the sum of doubled DBP and SBP divided by 3:

$$MAP = (SBP + 2 \times DSP) \div 3$$

Figure 2.7 Figure shows automated heart pressure and heart rate Omron device

2.4.3.7.4 Non-invasive measurement of cardiac output (CO)

Measurements of cardiac parameters were obtained noninvasively by taking measurements with a gas rebreathing method – Innocor® and standard bioimpedance approach (Cheetah, NICOM). The cardiovascular parameters that were recorded or calculated were cardiac output (CO), stroke volume (SV), cardiac index (CI), stroke volume index (SVI), heart rate (HR) and peripheral vascular resistance (PVR).

Cardiac output is the volume of blood that the heart pumps per minute. It is calculated by multiplying the stroke volume (SV) and heart rate (HR). The normal range for CO is 4–8 L/min but this can vary from person to person. Stroke volume (SV) is the volume of blood pumped out of the heart in one beat usually ranging 60–100 ml/beat. It is determined by preload, cardiac contractility and afterload.

$$CO\ (L/min) = HR\ (beat/min) \times SV\ (ml/beat) \div 1000$$

Preload is the amount of ventricular stretch at the end of diastole. Afterload is known as peripheral vascular resistance (PVR) which reflects the changes in arterioles and is the amount of resistance the heart must overcome to open the aortic valve and push the blood into systemic circulation. PVR was calculated as MAP divided by CO and multiplied by 80. Normal PVR is 700 to 1500 dynes/sec/cm^{-5}.

$$PVR\ (dynes/sec/cm^{-5}) = MAP\ (mmHg) \times 80 \div CO\ (L/min)$$

Cardiac index (CI) is an assessment of the CO value based on participant's body surface area (BSA) and is calculated by dividing CO and BSA. Normal range is 2.5–4 L/min/m^2. Body surface area measures the total surface area of the body and is defined by the Mosteller formula which is a square root of multiplied weight measured in kilograms and height measured in centimetres and divided by 3600.

$$CI\ (L/min/m^2) = CO\ (L/min) \div BSA\ (m^2)$$

Stroke volume index (SVI) is the volume of blood in millilitres (ml) pumped by the heart with each beat divided by the BSA, usually 33–47 ml/m^2/beat.

$$SVI\ (ml/m^{2/}beat) = SV\ (ml/beat) \div BSA\ (m^2)$$

2.4.3.7.4.1 Gas rebreathing method – Innocor®

Innocor gas rebreathing method (Innovision A/S, Odense, Denmark) measures the levels of two inert gases in the participant's mouth over the course of about six breaths and by detecting changes in these gas concentrations calculates CO, CI and SV (Middlemiss *et al.,* 2019).

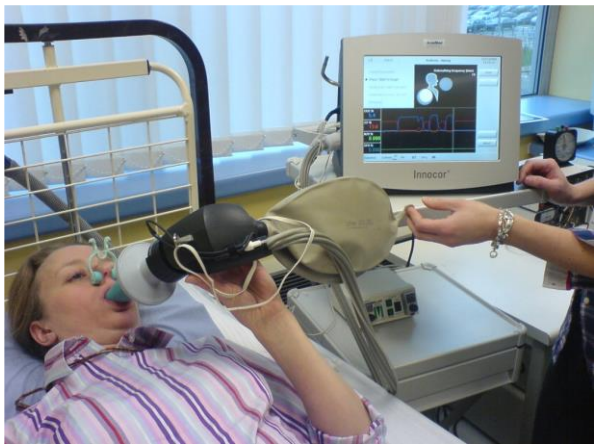
The rebreathing bag is prefilled with an O_2 enriched mixture typically containing 0.5 % nitrous oxide (N_2O) as inert blood soluble and 0.1 % sulphur hexafluoride (SF_6) as inert blood insoluble gas. Levels of these gases are measured continuously in the participant's mouth by a fast-responding infrared gas analyser inside Innocor®.

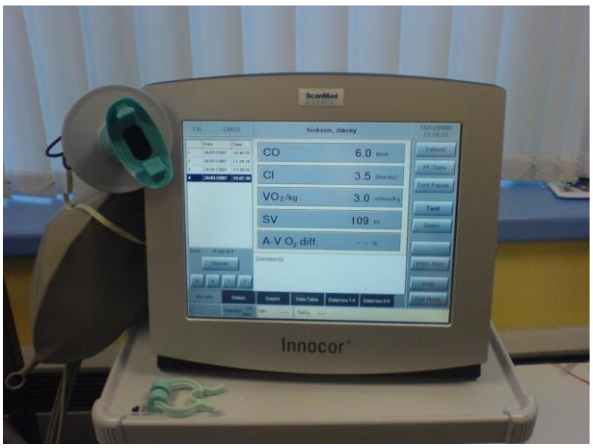
The method relies on the principle that the concentration of the soluble gas (N_2O) falls during rebreathing due to solution of the gas in blood and lung tissue. Rate of its disappearance from the alveolar space is then proportional to the flow of blood perfusing the ventilated parts of the lungs (effective pulmonary blood flow). This is equal to the CO in the absence of a significant intrapulmonary shunt. The blood insoluble gas (SF_6) is measured to determine the lung volume from which the soluble gas disappears. Since the volume of the breathing bag is known, the total lung volume can be determined from the dilution of the insoluble gas.

Participants were lying supine with their head supported by pillows in a quiet, temperature controlled (23–25 °C) room for the duration of the study. A pulse oximeter probe was placed on the left index finger, nose clip on participant's nose and whilst measurements were taken a mouth piece was inserted (Figure 2.8 a). Participants were asked to take deep breaths at a normal respiratory rate (around 20 breaths/per minute) guided by the speed indicator shown on the screen. Once an adequate breathing rhythm was achieved, measurements were taken for the following six breaths ensuring that the bag was completely emptied by each breath.

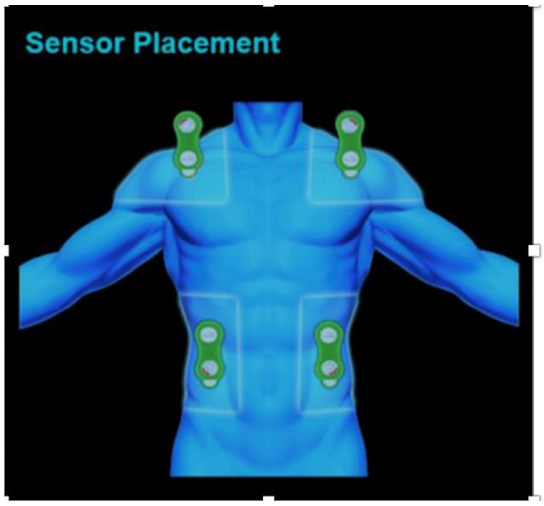
The method was solely used in Study 1, the reasons for which will be explained under the Section 3.8. CO, CI and SV were measured at baseline and then every 30 minutes until the end of infusion.

Figure 2.8 Figure shows methods used for non-invasive measurements of cardiac output.





a) Participant using Innocor gas rebreathing method. Courtesy of Dr Carmel McEniery



b) Placement of Cheetah sensors and Starling SV monitor in bioreactance-based CO measurements. Pictures were selected from Cheetah manual provided with equipment

2.4.3.7.4.2 Bioreactance-based noninvasive CO measurement

The Cheetah NICOM Reliant System version 5.2 (Cheetah Medical Ltd, Tel Aviv, Israel) was used for all three Studies. It is a portable, non-invasive continuous hemodynamic monitoring device based on the principle of bioreactance. The system is comprised of Cheetah Sensors™ (in further text sensors) and a Starling SV monitor (in further text monitor) as shown in Figure 2.8 b.

The sensors have double-electrodes, which carry out two simultaneous roles. At first, they deliver the low amplitude alternating current induced by the monitor. The second sensor role is to detect changes in voltages after the current passes through the thorax. The monitor detects the time delay between input and output using a phase detector.

This standard bioimpedance approach relies on detecting changes in electrical impedance to the passage of a low electrical current of known amplitude within the thorax to measure the change in voltage at the receiving electrodes. Both blood and body tissue impede electrical current, but the volume and impedance of the solid tissues remains constant during the cardiac cycle. The volume of thoracic fluid (mostly in the aorta and other intrathoracic large arteries), however, changes with each cardiac beat. Changes in intrathoracic fluid volume change both bioimpedance and bioreactance. Transthoracic impedance is the change in amplitude of applied voltage and bioreactance is the time delay (or phase shift) between applied signal relative to the received signal, which correlates with stroke volume and cardiac output (Keren *et al.,* 2007).

After these signals are analyzed, the computer measures HR and calculates SV and CO, whilst by using height and weight it calculates body surface area used for CI and SVI measurements.

As described in Section 2.4.2.2 volunteers were asked to lie supine with their head supported by pillows in a quiet, temperature controlled (23–25 °C). Four single use prewired sensors with symmetrical right-left placement were used in each study and placed following the manufacturer's guidance; two upper sensors were positioned above the heart, on the right and left supraclavicular, whilst two lower ones were positioned below the heart, on right and left costal margins (Figure 2.8 b). During automatic calibration subjects were asked to remain still and quiet. Following calibration, the NICOM recorded cardiac parameters ~~at~~ as an average 1-minute intervals–for the duration of the study. Each measurement was the mean of the preceding one-minute data.

The parameters recorded by Cheetah Nicom were CO, CI, SV, SVI and HR. Haemodynamic recordings in the resting state started 20 minutes prior to the infusion start and were taken at 10-minute intervals. In Study 1 recordings were done at baseline and 30, 60, 90 and 120 minutes into the infusion, whilst in Studies 2 and 3 after baseline, measurements were continued at 5 minutes intervals in the first hour and 15-minute intervals in the second hour.

2.5 Assessment of safety

An assessment of safety was done throughout the study based on clinical review and collected laboratory and hemodynamic parameters, from the point of participant signing the Informed Consent, regardless of whether a participant has yet received any treatment.

Adverse event (AE) and serious adverse events (SAE) were defined per standard GCP definitions and monitored and recorded throughout the study.

2.6 Data handling

All data were transferred into a Case Report Form which were anonymized and completed by myself (study doctor) and kept for the records together with original signed informed consent, medical records, 12 lead ECG, blood results (electronic or paper) and sample logs.

2.7 Statistical analyses

2.7.1 Sample size

Statistical power calculations were used to determine the sample sizes for Studies 2 and 3 in order to test the hypothesis in a sufficiently powered cohort.

2.7.1.1 Study 1

This was a series of dose ranging pilot studies to dissect tolerability and safety in three [Pyr^{1}]apelin-13 doses, therefore, no formal sample size calculation was performed. Sample size of 6 participants was guided by results and safety observations of previous research (Japp *et al.,* 2008; Japp *et al*., 2010; Barnes *et al.,* 2013; Gourdy *et al.,* 2018).

2.7.1.2 Study 2

Sample size power calculations were based on previously published work (Japp, *et al.,* 2010). A sample size of 8 participants was calculated to enter this 2-treatment study with 92 percent probability that the study will detect 0.5 L/min/m^2 increase in CI with standard deviation of 0.25 L/min/m^2 at a two-sided 0.05 significance level. Study sample size was increased to a total of 9 participants in each dose group (18 in total) to ensure quantity of data are sufficient if a participant does not complete the study.

2.7.1.3 Study 3

Sample size of 9 participants was chosen to match sample size described under Study 2 (2.7.1.2).

2.7.2 Data analysis

Data were recorded in a blinded fashion for Studies 2 and 3 and unblinded for Study 1. An interim analysis of data was performed after Study 1 and Study 2 to assess the effects of [Pyr^{1}]apelin-13 and to determine the doses to be used in subsequent studies. Student performed the data analysis under guidance of Dr Kaisa Maki Petaja. Analyses were done when each study was completed and unblinded (in Studies 2 and 3). Values were log transformed before statistical analyses if not normally distributed. Shapiro-Wilk test and normality plots (Q-Q) were used to determine the distribution of the data. N was the number of participants. Variables are reported as mean±SE and analysed with 2-way repeated measures ANOVA using SPSS version 26 (IBM, New York, USA) with *posthoc* Bonferroni corrections and independent 2-tailed Student's t-test to compare differences between groups of subjects. Statistical significance was taken at the 5 % level.

2.8 Supervision and collaboration

Study has been conducted in collaboration of AstraZeneca and EMI (Experimental medicine and immunotherapeutics) at Cambridge University Hospital. Student (Dr Petra Sulentic) designed clinical study protocol and has undertaken necessary regulatory submissions (Section 2.1.1) under supervison of Prof. Anthony Davenport (academic supervisor), Dr Joseph Cheryian (clinical research supervisor) and Dr Phil Ambery (industry supervisor). As Student had to be blinded to the treatment, she has been supported by nursing staff in the Unit, who prepared study treatment for each dosing visit. Dr Janet Maguire and Dr Kaisa Maki Petaja advised Student towards the choice of statistical methods so Student could perform the analyses (Sections 2.7.2, 4.5 and 5.5). Venous blood samples have been analysed by Clinical Pathology and Core Biochemical Assay Laboratory at Cambridge University Hospital (Section 2.4.3.2).

Chapter 3:

Study 1 – Pilot, open label, dose ranging study to investigate safety and tolerability of increasing doses of [Pyr^{1}]apelin-13 in healthy volunteers

3.1 Background

3.1.1 Safety and tolerability of infused apelin peptide in clinical studies

A number of pre-clinical animal studies and human *in vitro* research identified the potential therapeutic roles of naturally occurring apelin peptide in the human cardiovascular system and in glucose homeostasis (described in detail in Section 1.3.2). To date, only a limited number of human *in vivo* interventional studies have been conducted, as listed in Table 1.1.

Apelin has been used in both Forearm Blood Flow (FBF) and systemic studies by ourselves (Brame *et al*., 2015; Yang *et al*., 2017) and other research groups (Japp *et al*., 2010; Barnes *et al*., 2013) as previously shown in Table 1.1. These studies confirmed apelin has a short half-life of less than 5 minutes (Japp *et al*., 2010; Barnes *et al*., 2013) (Section 1.3.1.3.2). Apelin administration was reported to be safe, without adverse effects in either HF patients or healthy participants in both FBF and systemic short-term and long-term infusions (Japp *et al.,* 2008; Japp *et al.,* 2010; Barnes *et al.,* 2013; Brame *et al.,* 2015, Gourdy *et al.,* 2018).

Apelin dose ranges in previous FBF studies were 0.3–100 nmol/min infused over 6 minutes (Brame *et al.,* 2015; Japp *et al*., 2010; Barnes *et al*., 2013). Doses used in systemic cardiovascular human studies ranged from 30–300 nmol/min administered over 5 to 10 minutes for each dose, and 30 nmol/min over a prolonged 6-hours infusion (Japp *et al.,* 2010; Barnes *et al.,* 2013). [Pyr^{1}]apelin-13 doses used in hyperinsulinemic euglycemic clamp were 9 nmol/kg and 30 nmol/kg administered through intravenous continuous infusion at the constant rate of 75 and 250 pmol/kg/min respectively during 2 hours (Gourdy *et al.,* 2018).

3.1.2 Study 1 dose testing approach

We predicted that an infusion duration of 2 hours would be sufficient not only to detect changes in cardiovascular parameters, but also to produce changes in insulin sensitivity.

The proposed Study 1 dose testing approach was designed to use initial concentrations of [Pyr^{1}]apelin-13 at 30 nmol/min for the first dose. This falls within the range previously shown to exert cardiovascular effects and to be safe (Barnes *et al.,* 2013). The second [Pyr^{1}]apelin-13 dose tested in Study 1 was planned at 90 nmol/min, representing equivalent to the cumulative dose of peptide administered in the Barnes studies with 30 nmol/min over 6 hours (Barnes *et al.*, 2013). If dosing remained within the safety protocol criteria, the [Pyr^{1}]apelin-13 dose on the third dosing visit was planned to be increased by 50 % to 135 nmol/min.

This was the first in man study to examine safety and tolerability of prolonged 2 hour systemic [Pyr^{1}]apelin-13 infusion in the highest doses reported to date in healthy participants.

3.2 Study aims, objectives and endpoints

3.2.1 Study aims

Study 1 aim was to test the proposed study methods, estimate the safety and tolerability of [Pyr^{1}]apelin-13 in three increasing doses in healthy volunteers and to determine the [Pyr^{1}]apelin-13 doses likely to produce cardiometabolic effects in Studies 2 and 3.

3.2.2 Study objectives

The Study objective was to define a range of safe and tolerable doses of intravenous [Pyr^{1}]apelin-13 which may have an effect on glucose homeostasis and cardiovascular physiology.

3.2.3 Study endpoints

The Study endpoints after infusion of [Pyr^{1}]apelin-13 were to detect:

1. Changes in parameters evaluating safety and tolerability including BP, HR, POC glucose, ECG, cardiac telemetry
2. Changes in markers of glucose homeostasis including glucose, C-peptide, insulin
3. Changes in parameters of cardiovascular haemodynamics including cardiac index measured by gas rebreathing method and bioreactance

3.3 Study hypothesis

The hypotheses stated that:

1. intravenously infused [Pyr^{1}]apelin-13 will be safe and tolerable in all three increasing doses tested in healthy volunteers
2. intravenously infused [Pyr^{1}]apelin-13 will increase cardiac index (CI) in healthy volunteers

These hypotheses were tested with systemic intravenous infusions of [Pyr^{1}]apelin-13, blood sampling and non-invasive cardiovascular measurements (cardiac output measurements, cardiac telemetry, ECG, vital signs measurements)

3.4 Methods

Pilot Study 1 was an open label study in healthy volunteers. In total nine participants underwent screening and six met the study entry inclusion criteria.

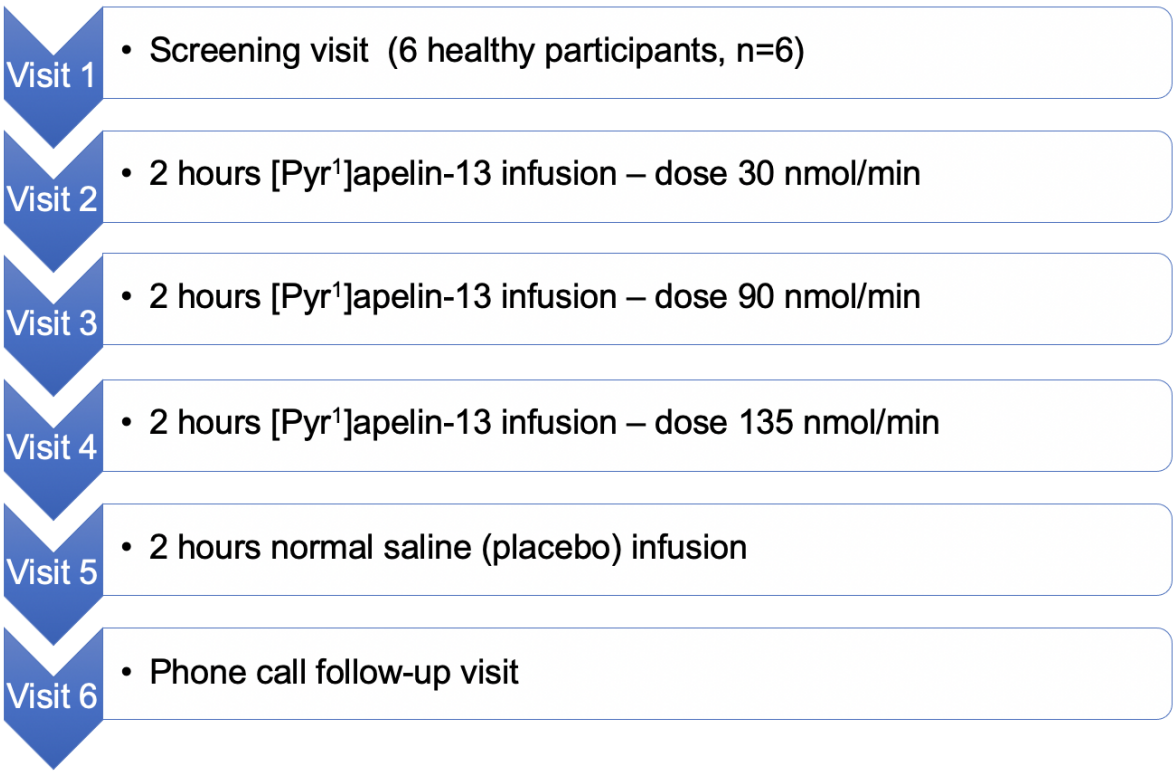
Inclusion Criteria:

- BMI measured 18–27 kg/m^2 with either one of the criteria below
    - waist circumference less than 88cm (35 inches) for women or 102 cm (40 inches) for men
    - and/or body fat levels less than of 32 % for women and 25 % for men
- current non-smoker
- age range 18–70 years

Exclusion criteria were previously listed in Chapter 2 (section 2.2.2.2).

The general methods for systemic studies and participants restrictions were outlined in detail in Chapter 2. Each participant attended four dosing visits on separate days as is shown in Figure 3.1 Adverse events (AE) were noted and cardiovascular and metabolic variables were measured at predefined times during infusion as outlined in Chapter 2 (Sections 2.4.3.7 and 2.4.3.2, 2.5) and is shown in Figure 3.2.

Figure 3.1 Study 1 protocol



Each of the 6 participants attended screening (V1) and four dosing visits (V2–V5). All participants were allocated to the same 2 hours [Pyr[1]]apelin-13/normal saline dosing schedule with increasing doses of [Pyr[1]]apelin-13; 30, 90 and 135 nmol/min on Visit 2, 3, and 4 respectively and placebo (normal saline) on Visit 5. They received a phone call as part of Visit 6.

Figure 3.2 Schedule of assessments in dosing visits for Study 1

	120 minutes [Pyr[1]]apelin-13 or normal saline infusion						
Timepoint / min	**0**	**15**	**30**	**45**	**60**	**90**	**120**
Cardiac telemetry	×	×	×	×	×	×	×
ECG	×						×
BP	×		×		×	×	×
HR	×		×		×	×	×
POC glucose	×	×	×	×	×	×	×
Bioreactance measurement	×		×		×	×	×
Gas rebreathing method	×		×		×	×	×
Venous samples	×	×	×	×	×	×	×

On each dosing visit participant underwent 120-minute infusion of [Pyr[1]]apelin-13/placebo after 20-minute resting interval. Cardiovascular and metabolic assessments were taken at predefined infusion time points. Cardiac telemetry recording was measured continuously throughout the study. Electrocardiogram recording (ECG) was taken at baseline and after infusion completion. Bioreactance-based and gas rebreathing method cardiac measurements, blood pressure (BP) and heart rate (HR) were taken at baseline and every 30 minutes into the infusion. Point of care (POC) glucose and venous blood samples for glucose, insulin and C peptide were taken at baseline and every 15 minutes in the first hour and every 30 minutes in the second hour of the infusion.

3.5 Data Analysis

This was a pilot study in healthy volunteers with sample size previously explained in Chapter 2 (2.7.1.1).

Demographic and baseline cardiovascular and metabolic participants' data are given as means±SD and analysed using Excel software (Microsoft Office). Data were normally distributed. N values are the number of participants who underwent study visits.

As this was an open label study, primary endpoint data for evaluating safety and tolerability (section 3.2.3) were clinically monitored throughout each dosing visit and descriptively noted without formal statistical analyses. Data for reporting changes in markers of glucose homeostasis and parameters of cardiovascular haemodynamic reported as secondary endpoints (section 3.2.3) are given as means±SE. Analyses were performed by two-way repeated measures ANOVA (SPSS version 26; IBM, New York, USA). Custom hypothesis testing (simple) of within-subject contrasts was performed for the [Pyr^{1}]apelin-13 in each dose *versus* normal saline comparison. Statistical significance was taken at the 5 % level. Greenhouse-Geisser corrected *P*-values were used if Mauchly's test revealed a violation of sphericity.

3.6 Study 1 results

The results of interim analyses for evaluation of metabolic and cardiovascular effects were used to guide dose selection used in Study 2.

3.6.1 Subjects characteristics

Baseline demographics, clinical, metabolic and cardiovascular characteristics of participants are shown in Table 3.1. Mean age was 43±16.8 years and participants were matched by gender. Four participants were white, one black and one Asian. All of them had active lifestyle and were not taking any medications at the time of the Study.

Table 3.1 Baseline demographic, cardiovascular and metabolic characteristics of Study 1 participants

Study 1	**Healthy participants**
Sample size (n)	6
Age (years)	43.8±16.8 [22–67]
Gender, M/F	3 M, 3 F
Ethnicity W/B/A	4 W, 1 B, 1 A
BMI (kg/m^2)	23±2.4 [19.7–26.5]
Waist circumference (cm)	77±5.7 [71–85]
Body fat mass (%)	21.4±9.4 [8.6–33.5]
Ex-smokers, n	0
Medication, n	0
Baseline	
HR, bpm	63±13.7 [45–85]
MAP, mm Hg	80.3±3.9 [75–85.7]
PVR, dynes/sec/cm^{-5}	1262.3±324.7 [925.7–1837.1]
CI, L/min/m^2	3.1±0.5 [2.1–3.5]
HbA1c (mmol/mol)	36±2.1 [34–39]

Gender; M – Male, F – Female. Ethnicity; W – White, B – Black, A – Asian. BMI – Body Mass Index. HR – Heart Rate. MAP – Mean Arterial Pressure. PVR – Peripheral Vascular Resistance. CI – Cardiac Index. HbA1c – Glycated Haemoglobin. $HOMA_{IR}$ – Homeostatic Model Assessment for Insulin Resistance. Values are represented as means ± standard deviation and [range].

3.6.2 Safety and tolerability study results

All participants completed four dosing visits in full. All three [Pyr[1]]apelin-13 doses infusions were found to be well tolerated without serious adverse events (Table 3.2) and participants remained haemodynamically stable without clinically significant ECG changes (Table 3.3).

3.6.2.1 Tolerability of [Pyr[1]]apelin-13 infusions in increasing doses

Throughout the Study 1 there were altogether four mild adverse events (AE) as shown in Table 3.2. Three AE described as mild subjective feeling of nasal congestion occurred on second and third dosing visit when 90 nmol/min and 135 nmol/min [Pyr[1]]apelin-13 doses were infused respectively. AEs were in probable relation to the study as they lasted until the end of infusion completion and spontaneously resolved within minutes afterwards.

Table 3.2 Reported adverse events (AEs) in Study 1

AE severity	[Pyr[1]]apelin-13 dose	Side effect	Causality	Events, n
Mild	30 nmol/min	common cold	not related	1
	90 nmol/min	nasal congestion	probably related	1
	135 nmol/min	nasal congestion	probably related	2

3.6.2.2 Safety of [Pyr[1]]apelin-13 infusions in increasing doses.

None of the participants met study dosing visits stopping criteria (see Section 2.1.4) and Table 3.3.

3.6.3 Cardiovascular and metabolic measurements results

The effects of two-hour [Pyr[1]]apelin-13 infusions (V2-V4) compared to normal saline (V5) on cardiovascular and metabolic parameters are shown in Table 3.6. Prolonged 2 hour [Pyr[1]]apelin-13 infusion in three escalating doses (30, 90, 135 nmol/min) does not cause overall significant change in cardiovascular and metabolic parameters. Significantly different from saline control $P \leq 0.05$. Cardiovascular and metabolic data are given in Table 3.4 and 3.5.

Table 3.3 Study 1 dosing visit safety evaluation parameters (V2–5)

Treatment	variable (mean±SD)	time (min)						
		0	15	30	45	60	90	120
V2-apelin 30 nmol/min	HR (beats/min)	63.8±16	-	59.6±11.9	-	59.5±11.2	60.3±10	61.3±11.3
	MAP (mmHg)	80.6±10.3	-	82.9±9.9	-	82.2±9.9	85.7±10.3	86±13
	SBP (mmHg)	107.2±16.4	-	110.17±15	-	109.9±13.3	115.7±16.2	115±16.4
	DBP (mmHg)	67.3±8.3	-	69.3±7.8	-	68.3±9.1	70.7±8	71.5±12.1
	glucose (mmol/L)	4.7±0.8	4.7±0.7	4.6±0.6	4.6±0.8	4.6±0.9	4.5±0.8	4.5±0.7
	ECG	normal	no change	no change	no change	no change	no change	no change
	telemetry	normal	no change	no change	no change	no change	no change	no change
V3-apelin 90 nmol/min	HR (beats/min)	62.5±13.1	-	57.3±9.8	-	59.3±11.4	59.5±11.6	60±12
	MAP (mmHg)	78.1±8.2	-	81.5±10.9	-	81.9±9.2	83.4±9.5	84.1±9.4
	SBP (mmHg)	105±12.3	-	109.5±15.1	-	111.5±13.3	113.8±16.5	114.5±15.3
	DBP (mmHg)	64.7±6.8	-	67.5±9.2	-	67.2±8.3	68.2±7.1	68.8±7.3
	glucose (mmol/L)	4.1±0.5	4.2±0.4	4.3±0.4	4.3±0.4	4.3±0.3	4.2±0.3	4.4±0.3
	ECG	normal	no change	no change	no change	no change	no change	no change
	telemetry	normal	no change	no change	no change	no change	no change	no change

Treatment	variable (mean±SD)	time (min)						
		0	15	30	45	60	90	120
V4-apelin 135 nmol/min	HR (beats/min)	61.7±8.2	-	57±9.9	-	59.7±9.9	59.2±9.8	59.5±8.2
	MAP (mmHg)	79.8±7.9	-	81.2±8.9	-	82.7±9.3	81.3±6.9	82.6±9.6
	SBP (mmHg)	109±11	-	109.3±10.6	-	112±9.9	110.8±7.7	112.8±9.5
	DBP (mmHg)	65.2±7.5	-	67.2±9.3	-	68±10.1	66.5±8	67.5±10.6
	glucose (mmol/L)	4.2±0.7	4.2±0.7	4.2±0.6	4.3±0.3	4.4±0.2	4.3±0.4	4.5±0.2
	ECG	normal	no change	no change	no change	no change	no change	no change
	telemetry	normal	no change	no change	no change	no change	no change	no change
V5 Normal saline	HR (beats/min)	61.8±13.5	-	62±13.1	-	64±10.7	64.7±13.1	64.7±11.4
	MAP (mmHg)	80.3±3.9	-	84.5±7.8	-	82.3±12.4	85.8±8.6	87.3±11.8
	SBP (mmHg)	107.8±9.9	-	116.2±10.1	-	116.8±12.8	115.3±13.3	119.2±16.6
	DBP (mmHg)	66.5±4.2	-	68.7±7.5	-	65±13.2	71±6.8	71.3±9.9
	glucose (mmol/L)	4.6±0.4	4.5±0.3	4.4±0.3	4.4±0.3	4.4±0.3	4.4±0.4	4.5±0.4
	ECG	normal	no change	no change	no change	no change	no change	no change
	telemetry	normal	no change	no change	no change	no change	no change	no change

At each dosing visit the participant received an infusion of one of the following; [Pyr^{1}]apelin-13 at 30, 90 or 135 nmol/min or normal saline (control). Mean data for each participant from the four visits is presented. HR; heart rate. MAP; mean arterial pressure. SBP; systolic blood pressure. DBP; diastolic blood pressure. ECG; electrocardiogram.

Table 3.4 Study 1 dosing visit (V2-V5) cardiovascular parameters

Treatment	variable (mean±SD)	time (min)						
		0	15	30	45	60	90	120
V2-apelin 30 nmol/min	CI (L/min/m^2)	3.2±0.75	-	3.2±0.46	-	2.95±0.48	3.1±0.43	3.15±0.64
	SVI (ml/m^{2}/beat)	50.5±10.55	-	52.66±8.47	-	48.83±9.36	50.66±8.40	49.5±10.3
	PVR (dynes/sec/cm^{-5})	1277±393	-	1237±194	-	1348±279	1317±232	1334±341
V3-apelin 90 nmol/min	CI (L/min/m^2)	3.35±0.51	-	3.5±0.46	-	3.23±0.57	3.23±0.5	3.31±0.57
	SVI (ml/m^{2}/beat)	54.6±12.9	-	46.4±19.8	-	55.2±11.6	53.8±11.4	55.5±12.8
	PVR (dynes/sec/cm^{-5})	1117±181	-	1112±155	-	1233±249	1242±250	1241±328
V4-apelin 135 nmol/min	CI (L/min/m^2)	3.78±0.36	-	3.6±0.68	-	3.28±0.67	3.1±0.6	3.03±0.43
	SVI (ml/m^{2}/beat)	63.2±9.5	-	61.7±10.3	-	57.2±11.2	52.8±9.7	51.5±9.1
	PVR (dynes/sec/cm^{-5})	1012±227	-	1098±241	-	1240±338	1292±374	1321±403
V5 Normal saline	CI (L/min/m^2)	3.08±0.51	-	3.05±0.55	-	3.03±0.62	3.08±0.58	3.08±0.55
	SVI (ml/m^{2}/beat)	52.2±15.2	-	48.8±12.1	-	48±15.2	49.3±15	47.7±14.4
	PVR (dynes/sec/cm^{-5})	1262±324	-	1348±394	-	1343±481	1370±438	1378±417

At each dosing visit (V2-V5) participant received a 2 hour infusion in consecutive order; [Pyr^{1}]apelin-13 at 30, 90 or 135 nmol/min and normal saline (control). Mean data for each participant from the four visits is presented. Mean HR, MBP, DBP and SBP are presented in Table 3.3. CI; cardiac index. SVI; stroke volume index. PVR; peripheral vascular resistance.

Table 3.5 Study 1 dosing visit (V2-V5) metabolic parameters

Treatment	variable (median, [IQR])	time (min)						
		0	**15**	**30**	**45**	**60**	**90**	**120**
V2-apelin 30 nmol/min	insulin (pmol/L)	28 [21-58]	24 [20-50]	23.5 [17-37]	45 [22-66]	18.5 [13-51]	18.5 [16-46]	14 [14-27]
	C peptide (pmol/L)	735 [478-992]	651.5 [455-954]	582 [440-853]	525 [369-832]	471 [382-807]	424.5 [348-749]	384.5 [381.5-755]
V3-apelin 90 nmol/min	insulin (pmol/L)	22.5 [11.3-36]	28.5 [15.7-37.5]	29 [8-29]	26 [10-31]	19.5 [14.2-31.5]	17 [10.5-23.5]	15.5 [10.7-18.7]
	C peptide (pmol/L)	501.5 [475-609]	496 [480-566]	441.5 [398-528]	427.5 [368-473]	432.5 [399-456]	387 [336-410]	339 [301-360]
V4-apelin 135 nmol/min	insulin (pmol/L)	37 [29-93]	22 [18-32]	31 [19-45]	33 [20-38]	24.5 [18.5-32]	22 [14.7-30.7]	20.5 [15.2-25.7]
	C peptide (pmol/L)	596.5 [504-962]	514 [477-754]	506.5 [470-754]	504 [454-715]	475.5 [442-648]	473.5 [411-551]	422 [413-547]
V5-normal saline	insulin (pmol/L)	23.2 [14-64]	21.7 [14-38]	18.5 [14-33]	17.9 [13.7-32.2]	17.9 [13.9-35.7]	18.7 [15.9-27.6]	19.3 [17.9-27.9]
	C peptide (pmol/L)	476 [422-767]	430 [408-620]	417 [403-582]	393 [388-548]	390 [374-553]	394 [376-514]	376 [365-470]

At each dosing visit (V2-V5) participant received 2 hour infusions in consecutive order; [Pyr^{1}]apelin-13 at 30, 90 or 135 nmol/min and normal saline (control). Insulin and C peptide data were not normally distributed and are presented as median and interquartile range [IQR].

Table 3.6 Cardiovascular and metabolic effects of two-hour [Pyr[1]]apelin-13 infusions in Study 1 participants

Visit	Treatment	Dose	Variable	*P* value
V2	[Pyr[1]]apelin-13	30 nmol/min	CI	0.64
			SVI	0.60
			HR	0.48
			PVR	0.39
			MAP	0.95
			glucose	0.79
			Insulin	0.51
			C peptide	0.73
V3	[Pyr[1]]apelin-13	90 nmol/min	CI	0.63
			SVI	0.35
			HR	0.07
			PVR	0.70
			MAP	0.93
			glucose	0.10
			Insulin	0.29
			C peptide	0.28
V4	[Pyr[1]]apelin-13	135 nmol/min	CI	0.10
			SVI	0.3
			HR	0.8
			PVR	0.12
			MAP	0.56
			glucose	0.42
			Insulin	0.48
			C peptide	0.9

Bioreactance cardiovascular (CI, SVI, HR, PVR, MAP) and metabolic (glucose, insulin, C peptide) effects are recorded on four dosing visits (V2–V4) following systemic two hour [Pyr[1]]apelin-13 infusion at increasing doses; 30, 90 and 135 nmol/min. Effects in each dose (V2-V4) are compared with the normal saline control (V5) by 2-way ANOVA repeated measures. There was no omnibus significance noted for any of the variables. HR – Heart Rate. MAP – Mean Arterial Pressure. PVR – Peripheral Vascular Resistance. CI – Cardiac Index. SVI – Stroke Volume Index. Data are presented as mean ± SE n=6. *gas rebreathing data are not presented

3.7 Study limitations

Throughout the study it was not possible to collect all of the gas rebreathing readings because of the technical reasons. On several occasions, the machine failed to produce readings and some of the participants found it challenging to produce technically good breaths which limited further formal statistical analyses.

In addition, formal calculation of $HOMA_{IR}$ could not have been performed as some of the insulin samples were partially haemolysed which affected the credibility of the result.

In this Study, we tried measuring apelin serum concentrations in collaboration with Core Biochemical Assay Laboratory (CBAL). Despite tested apelin kit was run according to the manufacturer's instructions (Section 2.4.3.2), its performance failed both in terms of sensitivity and precision. Issue has been raised with the manufacturer but has never been followed up with the response (explanation with courtesy of Peter Barker, senior scientist at CBAL).

3.8 Discussion

These pilot, open label studies were designed to assess *in vivo* safety, tolerability and cardiometabolic effects of apelin receptor agonism with [Pyr^1]apelin-13 in three increasing doses. We demonstrated all three [Pyr^1]apelin-13 doses (30, 90 and 135 nmol/min) to be safe and well tolerable. However, results regarding apelin cardiovascular and metabolic actions have not been fully conclusive, mainly due to the choice of CO measurement methods tested in these pilot studies.

Apelin has a good safety profile and is suitable for human *in vivo* experimental studies for two reasons. Firstly, apelin is a naturally occurring human body peptide and secondly, it is inducing effects which are short lived due to the quick proteolytic degradation (Japp *et al*., 2010; Barnes *et al*., 2013). Discoveries described by others (Japp *et al*., 2010; Barnes *et al*., 2013; Gourdy *et al.*, 2018) did not raise concerns regarding tolerability and safety of [Pyr^1]apelin-13 and our findings have been in agreement with previously published works. Although the doses used in this Study were 4-fold higher than the ones previously used in published cardiovascular research, there were no major subjective or objective recorded adverse events. Three volunteers who were receiving [Pyr^1]apelin-13 at medium and high doses (90 nmol/min and 135 nmol/min respectively) reported a subjective feeling of mild, nasal congestion, which promptly resolved within minutes upon [Pyr^1]apelin-13 infusion completion. In my opinion, such congestive feeling was most likely due to the vasodilating effects of apelin (Japp *et al.*, 2008; Japp *et al.*, 2010; Barnes *et al.,* 2013). In addition to the satisfying tolerability results, there were no safety concerns for any of the doses as all participants remained

haemodynamically stable with vital parameters fluctuating on average less than 5 percent off baseline, and without pathological changes of heart rhythm in ECG or cardiac telemetry.

Our selection of initial dose of [Pyr^{1}]apelin-13 dose at 30 nmol/min was mainly guided by Barnes *et al.* (2013) who reported sustained cardiovascular [Pyr^{1}]apelin-13 effects in prolonged 6 hours infusions in patients with chronic stable HF (Barnes *et al*., 2013). However, this Study has not proven similar cardiovascular effects in respect to any of the tested doses. Two non-invasive cardiac output measurement methods were used; gas rebreathing method (Innocor) and bioreactance method (Cheetah). Those techniques were tested as methods for measurement of cardiovascular parameters and the discrepancy in our results with previously published work (Japp *et al*., 2008; Japp *et al*., 2010; Barnes *et al*., 2013) can likely be attributed to several potential reasons, discussed below.

Prior to the start of the Study, it was predicted that there could be a potential impact of deep breaths, which are essential for gas rebreathing technique on cardiac preload. Therefore, Innocor measurements were taken at predefined time points, immediately following Cheetah measurements so that the effects of the expected interference in-between breathing and cardiac preload would be avoided. However, it remains possible that throughout the Study there was prolonged interference of deep breaths with cardiac preload, which may have after all affected the accuracy of bioreactance recordings set at 30 minutes intervals.

Despite the fact that the gas rebreathing method was fully explained and practised with all participants, outcomes remained dependant to the technique of the tested individual. Therefore, as some Innocor readings were reported incorrectly due to the suboptimal performance of the individual, this negatively affected the gas rebreathing data analysis.

Although other researchers have shown that the gas rebreathing method is not inferior to the bioreactance method in detecting changes in cardiovascular parameters (Middlemiss *et al.,* 2019), we decided to exclude it from further studies and to continue solely with the bioreactance method. The bioreactance method was easier to conduct and appeared more convenient for participants, hence it seemed to be a more suitable choice as the selected method for the detection of changes in CV parameters for further studies. In contrast to the gas rebreathing method, the bioreactance method can also detect rapid apelin effects appearing within minutes of the start of the infusion as it is producing measurements every minute.

As a result of the decision to use the bioreactance method over Innocor as a cardiovascular measurement technique, the protocols for Studies 2 and 3 had to be amended. The number of cardiovascular time points was increased with the intention of better understanding rapid

acting intravenous apelin actions in humans, which appear within minutes of the start of the infusion (Japp *et al*., 2008; Japp *et al*., 2010; Barnes *et al*., 2013)

We have not recorded any decrease in glucose indicative of hypoglycaemia, which was among the safety objectives of this pilot study. Unsurprisingly, in ANOVA statistical analyses we did not record results indicative of changes in insulin sensitivity as this was a group of fit and healthy participants without concomitant metabolic abnormalities and presumed to be insulin sensitive. This pilot study showed all tested doses were safe from a metabolic perspective and any dose could have been selected for further testing in the upcoming Studies 2 and 3. In addition, this Study has given us valuable insight that insulin measurements were severely influenced by even the slightest haemolysis, unlike C peptide measurements which were more stable and more reliable to process by the laboratory.

We acknowledge that this Study included small number of tested participants and has not given substantial amount of cardiovascular data especially due to technical issues caused by the use of two different cardiac output assessment methods.

In conclusion, [Pyr^{1}]apelin-13, at all three doses tested, was shown to be safe and tolerable and therefore can be tested in further studies. The cardiovascular effects of [Pyr^{1}]apelin-13 remain inconclusive following this pilot study, due to the small sample size and methodological issues. We have made changes to the protocol for Studies 2 and 3 and are going ahead using only the bioreactance method for the assessment of CO. We have also increased the frequency of time points in the protocol to achieve more reliable cardiovascular results in the upcoming studies. Based on our safety and tolerability data, we decided to use [Pyr^{1}]apelin-13 at dose 30 and 135 mol/min in the subsequent Study 2.

Chapter 4:

Study 2 – *In vivo* cardiovascular and metabolic assessment of [Pyr^{1}]apelin-13 prolonged infusions in participants with increased BMI

4.1 Background

Pre-clinical studies in isolated human tissue have shown apelin to be a potent endothelium-dependant arterial vasodilator and inotropic agent (Maguire *et al.*, 2009). In agreement, clinical studies conducted in Edinburgh showed a beneficial effect of systemically infused [Pyr^{1}]apelin-13 acting as a nitric oxide (NO) dependant arterial vasodilator, lowering mean arterial pressure (MAP), peripheral vascular resistance (PVR) as well as increasing cardiac output (CO) in both healthy subjects and in patients with heart disease (Barnes *et al.,* 2013; Japp *et al.*, 2008; Japp *et al.,* 2010). These actions of apelin were relatively short lived as the plasma half-life in humans is less than five minutes (Japp *et al.*, 2010). A recent study has demonstrated that [Pyr^{1}]apelin-13 improves insulin-stimulated endothelium-dependent and independent vasodilatation responses whilst reducing both Ang II and ET-1 induced vasoconstriction. This suggests that [Pyr^{1}]apelin-13 could improve haemodynamics in insulin resistant patients (Schinzari *et al.*, 2017).

In addition to beneficial cardiovascular actions, there is increasing evidence to demonstrate that apelin has a role in glucose metabolism. In a murine model, apelin-13 injection decreased glycemia (Dray *et al.*, 2008) and both [Pyr^{1}]apelin-13 and apelin-36 inhibited insulin secretion (Guo *et al.,* 2009; Sörhede Winzell *et al.,* 2005). Apelin-13 and [Pyr^{1}]apelin-13 directly increased insulin sensitivity by increasing glucose uptake of skeletal muscles and adipose tissue, determined during euglycemic-hyperinsulinemic clamp testing in mice (Dray *et al.,* 2008) and in overweight humans (Gourdy *et al.,* 2018). In addition, insulin resistance in apelin-deficient mice was reversed by exogenous [Pyr^{1}]apelin-13 treatment (Yue *et al.*, 2010). Furthermore, chronic apelin-13 treatment in mice contributed to improved insulin-stimulated glucose uptake in muscle and overall increased insulin sensitivity (Attané *et al.,* 2012).

These results suggest a significant role of apelin in the regulation of cardiovascular physiology and glucose homeostasis and are highlighting its therapeutic potential in obesity and other insulin resistance (IR) related diseases such as T2DM and diabetic cardiomyopathy. The apelin/APJ system is clearly of interest for therapeutic modulation as an agent with dual actions combining positive cardiovascular (positive inotropic effects and vasodilatation) and metabolic effects (modulation of insulin sensitivity). *In vivo* human studies are crucial to clarify cardiometabolic actions of [Pyr^{1}]apelin-13 and to establish a range of doses having measurable actions of apelin over two hours in a model population, before proceeding to targeted T2DM patients.

This is a first-in human study investigating the pharmacological properties of [Pyr^{1}]apelin-13 during systemic infusions in overweight/obese but otherwise healthy participants, serving as a model of IR. [Pyr^{1}]apelin-13 doses 30 and 135 nmol/min were selected based on safety and tolerability results of Study 1 (Section 3.6.2).

4.2 Study aims, objectives and endpoints

4.2.1 Study aims

Study 2 aims were to estimate the safety and tolerability of 2 hour [Pyr^{1}]apelin-13 infusion at dose 30 and 135 nmol/min in participants with increased BMI and to determine the [Pyr^{1}]apelin-13 dose likely to produce cardiometabolic effects in T2DM participants in Study 3.

4.2.2 Study objectives

The Study 2 objective was to define a range of safe and tolerable doses of intravenous [Pyr^{1}]apelin-13 which may have an effect on glucose homeostasis and cardiovascular physiology.

4.2.3 Study endpoints

Study endpoints after the infusion of [Pyr^{1}]apelin-13 at dose 30 and 135 nmol/min were to detect:

1. Changes in parameters evaluating the safety and tolerability including BP, HR, POC glucose, ECG, cardiac telemetry
2. Changes in markers of glucose homeostasis including glucose, C-peptide, insulin
3. Changes in parameters of cardiovascular haemodynamics including cardiac index measured by the bioreactance method

4.3 Study hypothesis

We tested for the first time the systemic cardiovascular effects and effect on insulin sensitivity of [Pyr^{1}]apelin-13 in participants with increased BMI in two [Pyr^{1}]apelin-13 doses (30 and 135 nmol/min respectively) with the following hypothesis:

1. intravenously infused [Pyr^{1}]apelin-13 will be safe and tolerable
2. intravenously infused [Pyr^{1}]apelin-13 will increase cardiac index (CI)
3. intravenously infused [Pyr^{1}]apelin-13 will increase insulin sensitivity

These hypotheses were tested with systemic intravenous infusions of the [Pyr^{1}]apelin-13, venous blood sampling and cardiovascular measurements (non-invasive cardiac output technology measurements, cardiac telemetry, ECG, vital signs measurements).

4.4 Methods

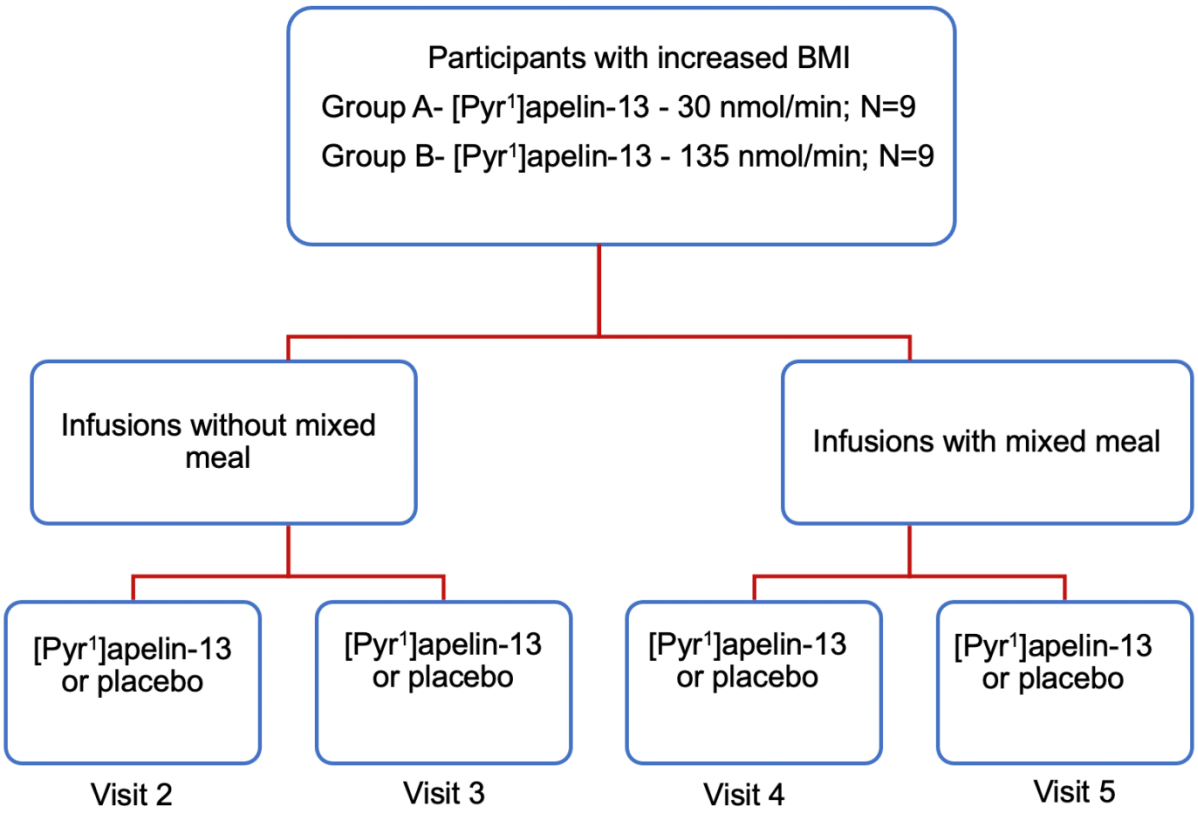
Study 2 was a randomized, double-blind study in participants with increased BMI. In total twenty-six participants underwent screening and eighteen met the study inclusion criteria (listed below). The choice of participants served as a model of insulin resistance, although this was not explicitly required for inclusion.

Participants study inclusion criteria:

- BMI measured 25–34.9 kg/m^{2} (WHO overweight/class I obese category) with either one of the criteria below
    - Waist circumference higher than 88cm (35 inches) for women or 102 cm (40 inches) for men
    - and/or body fat levels in excess of 32 % for women and 25 % for men
- Current non-smoker (not smoked within last 12 months)
- Age range 18–70 years

Exclusion criteria were previously listed in Section 2.2.2.2. The participants attended study visits as is shown in Figure 4.1 and outlined in Section 2.4.2.2. Each individual attended all dosing visits at the same time of the day. Adverse events (AE) were noted (Section 2.5) and cardiovascular and metabolic variables were measured at predefined times during infusion as was outlined in Chapter 2 (Sections 2.4.3.7 and 2.4.3.2) and is shown in Figure 4.2.

Figure 4.1 Protocol for Study 2.



Eighteen increased BMI participants were randomly allocated in two groups A and B of nine participants. Group A was infused with [Pyr[1]]apelin-13 in dose 30 nmol/min infusion and Group B received [Pyr[1]]apelin-13 in dose 135 nmol/min. Each participant was used as their own control, attending four double blind dosing visits with [Pyr[1]]apelin-13 or normal saline placebo administered in random order. Visit 2 and 3 were conducted without mixed meal (for measurement of cardiovascular parameters) and Visit 4 and 5 with mixed meal challenge (for measurement of post-prandial metabolic parameters). Mixed meal challenge is described in Section 2.4.3.5

Figure 4.2 Schedule of assessments in dosing visits for Studies 2 and 3.

	120 minutes [Pyr[1]]apelin-13 or normal saline infusion												
Timepoint / min	**0**	**5, 10**	**15**	**20, 25**	**30**	**35, 40**	**45**	**50, 55**	**60**	**75**	**90**	**105**	**120**
Cardiac telemetry	×	×	×	×	×	×	×	×	×	×	×	×	×
ECG	×												×
BP	×	×	×	×	×	×	×	×	×	×	×	×	×
HR	×	×	×	×	×	×	×	×	×	×	×	×	×
POC glucose	×		×		×		×		×	×*	×	×*	×
Bioreactance measurement	×	×	×	×	×	×	×	×	×	×	×	×	×
Venous samples	×		×		×		×		×	×*	×	×*	×
Mixed meal**	×												

On each dosing visit participants underwent a 120-minute infusion of [Pyr[1]]apelin-13 after a 20-minute resting interval (baseline). Cardiovascular assessments (bioreactance-based non-invasive cardiac measurements, blood pressure (BP) and heart rate (HR) were taken at baseline and in 5 minute intervals during the first hour of the infusion, followed by 15 minute intervals during the second hour. Cardiac telemetry was measured continuously throughout the study. An electrocardiogram recording (ECG) was taken at baseline and after infusion completion. Metabolic assessments (point of care [POC] glucose and venous blood samples for glucose, insulin and C peptide) were taken at time points 0, 15, 30, 45, 60, 90 and 120 minutes throughout the infusion. Mixed meal challenge was given on V4 and V5 in the first 5 minutes of infusion (Section 2. 4. 3. 5) * venous blood samples and POC glucose testing in Study 3 has been done every 15 minutes in the second hour of the infusion; at additional time points 75 and 105 minutes.

4.5 Data analysis

The sample size calculation for each group (A and B) of participants was described in Chapter 2 (Section 2.7.1.2). Data distribution was tested using Q-Q plots and Shapiro-Wilk test.

Demographic and baseline cardiovascular and metabolic participants' data are given as means±SD and analysed using Excel software (Microsoft office, IBM). Unpaired 2-tailed Student's *t*-tests were used to compare differences between two groups (A and B) of participants. A probability of $P<0.05$ was deemed significant.

N values are the number of participants who underwent study dosing visits. Cardiovascular and metabolic variables were reported for analyses of study endpoints in assessing the effects of [Pyr^{1}]apelin-13 infusions. Cardiovascular variables; CI, SVI, HR, MAP and PVR were analyzed in the first two dosing visits without mixed meal challenge (V2 and V3). Metabolic variables; glucose, insulin and C peptide were reported and analyzed in dosing visits with preceded mixed meal challenge (V4 and V5). Data are given as means±SE. For the skewed variables (insulin and C peptide) log-transformed values were used for the analyses. The significance was determined by 2-way ANOVA repeated measures (SPSS version 26; IBM, New York, USA), with *post hoc* testing with Bonferroni's adjustments. Data are graphically presented with Graphpad Prism (GraphPad Software Inc, USA) with help of Dr Janet Maguire. Greenhouse-Geisser corrected *P*-values were used if Mauchly's test revealed a violation of sphericity.

4.6 Study 2 results

4.6.1 Subjects characteristics

Data from nine participants in each group were obtained and baseline demographics, clinical, metabolic and cardiovascular characteristics of participants are shown in Table 4.1.

Participants in both groups were predominantly white and female. Although mean age of participants in the two dose groups was different, with Group A being on average younger (29.3±10.6 *versus* 44.6±17.4 years; $P<0.03$), BMI, cardiovascular variables, waist circumference and body fat percentage were not dissimilar between the groups. Both group participants had previous minimal smoking exposure with only one participant in each group having a smoking history of 0.05 and 0.25 pack-years respectively. Besides having increased BMI, participants were otherwise healthy and free of study relevant co-morbidities and medications as is shown in Table 4.2.

Table 4.1 Inter-group comparison of demographic, biochemical and baseline cardiovascular variables in group A and B participants with increased BMI

Study 2	Participants with increased BMI		
	Group A N=9	Group B N=9	*P*
Age (years)	29.3±10.6 [19–56]	44.6±17.4 [23–67]	0.03
Gender, M/F	3M, 6 F	2 M, 7 F	
Ethnicity, W/B/H	7 W, 1 B, 1 H	9 W	
BMI (kg/m^2)	29.8±2.1 [26.6–32.7]	29.1±3.3 [25.6–34.6]	0.6
Waist circumference (cm)	89.3±11 [68–103]	93.4±14 [78–117]	0.49
Body fat mass (%)	35.7±6 [29–44.3]	36.8±5.8 [23.3–43.2]	0.71
Ex-smokers, n	1	1	
Smoking (pack-years)	0.05	0.25	
Medications*	-	-	
Baseline cardiovascular			
HR, bpm	63.4±8.7 [50–77]	66.6±5.3 [58–74]	0.86
MAP, mm Hg	87.8±8.7 [77–105.3]	91.2±16.2 [72.7–114]	0.58
PVR, dynes/sec/cm^{-5}	958.3±151.1 [768.2–1186.7]	1054.4±409.7 [575.6–1900]	0.51
CI, L/min/m^2	3.8±0.3 [3.4–4.1]	3.9±0.6 [3–4.4]	0.69
HbA1c (mmol/mol)	34±3.1 [29–38]	34.9±4.9 [29–41]	0.65

Group A and B participants received [Pyr^1]apelin-13 infusion at dose 30 and 135 nmol/min respectively. Gender: M – Male, F – Female; Ethnicity: W – White, B – Black, H – Hispanic; BMI – Body Mass Index; HR – Heart Rate; MAP – Mean Arterial Pressure; PVR – Peripheral Vascular Resistance; CI – Cardiac Index; HbA1c – Glycated Haemoglobin; $HOMA_{IR}$ – Homeostatic Model Assessment for Insulin Resistance. ***** participants were not on regular medications relevant to the study.

Values are represented as means±SD and [range]. Unpaired 2-tailed Student's *t*-tests to evaluate for mean differences between groups. *P*-value <0.05 was deemed significant.

Table 4.2 Co-morbidities and concomitant medications in both groups of Study 2 participants

Medical condition	N	Medications
Arthritis	1	naproxen*****
Depression	2	venlafaxine
Atopic dermatitis	1	topical steroid cream
Asthma	1	salbutamol inhaler
Dyslipidaemia	1	simvastatin
Gastric reflux	1	omeprazole

***** regular treatments with anti-inflammatory medicines was part of exclusion study protocol criteria. As specified by study protocol naproxen was not taken 24 hours prior to the study visit

4.6.2 Cardiovascular and metabolic study results

Seventeen participants completed four study dosing visits in full. One of the participants in group B had to be excluded mid-study after completing the first two dosing visits. That event reduced N to 8 in Group B for Visits 4 and 5. The participant's exclusion was due to a Study unrelated adverse-event (Section 4.6.3).

4.6.2.1 Cardiovascular effects of 2-hour [Pyr^1]apelin-13 infusion in participants with increased BMI.

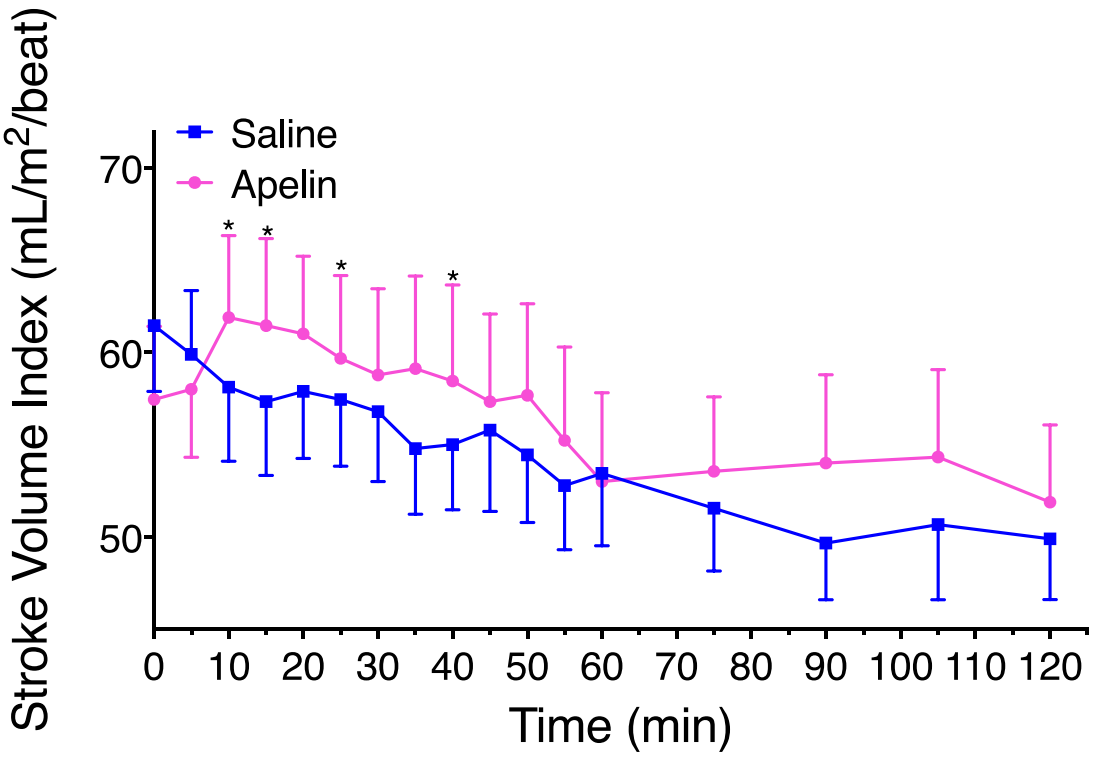
4.6.2.1.1 Changes in cardiovascular parameters after two hours of 30 nmol/min [Pyr^1]apelin-13 infusion in participants with increased BMI (group A).

Figures 4.3–4.9 represent changes in cardiovascular variables (SVI, CI, HR, MAP, SBP, DBP, PVR) in nine Study 2 participants (N=9) with increased BMI following systemic two hour [Pyr^1]apelin-13 infusion (30 nmol/min). Variables were measured on the first two dosing visits (V2 and V3) without mixed meal ingestion.

Continuous infusion of intravenous [Pyr^1]apelin-13 increased SVI over 2 hours, with most noticeable changes within the first 40 minutes, before their gradual return to baseline (ANOVA, omnibus P=0.002; Bonferroni post-test peak P=0.001 at 10 min; Figure 4.3). Cardiac index was overall significantly increased with post-hoc significant changes noted within first 15 mins (ANOVA, omnibus P<0.02; Bonferroni post-test peak P<0.001 at 10 mins; Figure 4.4). Heart rate increase reached borderline significant rise (ANOVA omnibus significance P=0.047; Figure 4.5) without showing significance in post-test Bonferroni analyses. MAP was overall reduced after 2 hours of [Pyr^1]apelin-13 infusion completion (ANOVA, omnibus significance P=0.002; Figure 4.6). PVR reduced as well, with a noticeable decline within the first 15 minutes, before a gradual return to baseline (ANOVA omnibus significance P=0.032; Bonferroni post-test P<0.001 at 10 mns time point; Figure 4.9). There were no overall significant changes in separate systolic and diastolic blood pressure measurements over the infusion period (Figure 4.7 and 4.8 respectively).

In summary, compared with a saline placebo control which had no effect on hemodynamic variables, systemic 2-hour [Pyr^1]apelin-13 administration caused significant rises in SVI (ANOVA P=0.002; Figure 4.3), CI (ANOVA P=0.02; Figure 4.4) and HR (ANOVA P=0.047; Figure 4.5) whilst reducing MAP (ANOVA P=0.002; Figure 4.6) and PVR (ANOVA P=0.032; Figure 4.9). *Post-hoc* testing with Bonferroni adjustments revealed [Pyr^1]apelin-13 CV effects were most notable after 10 minutes. Following the observed initial peak, CI and SVI declined back to baseline after 30 minutes, whilst MAP and PVR increased back to baseline after 20 minutes.

Figure 4.3 Effect of two-hour infusion of [Pyr[1]]apelin-13 (30 nmol/min) compared with 0.9 % saline control on stroke volume index (SVI) in participants with increased BMI

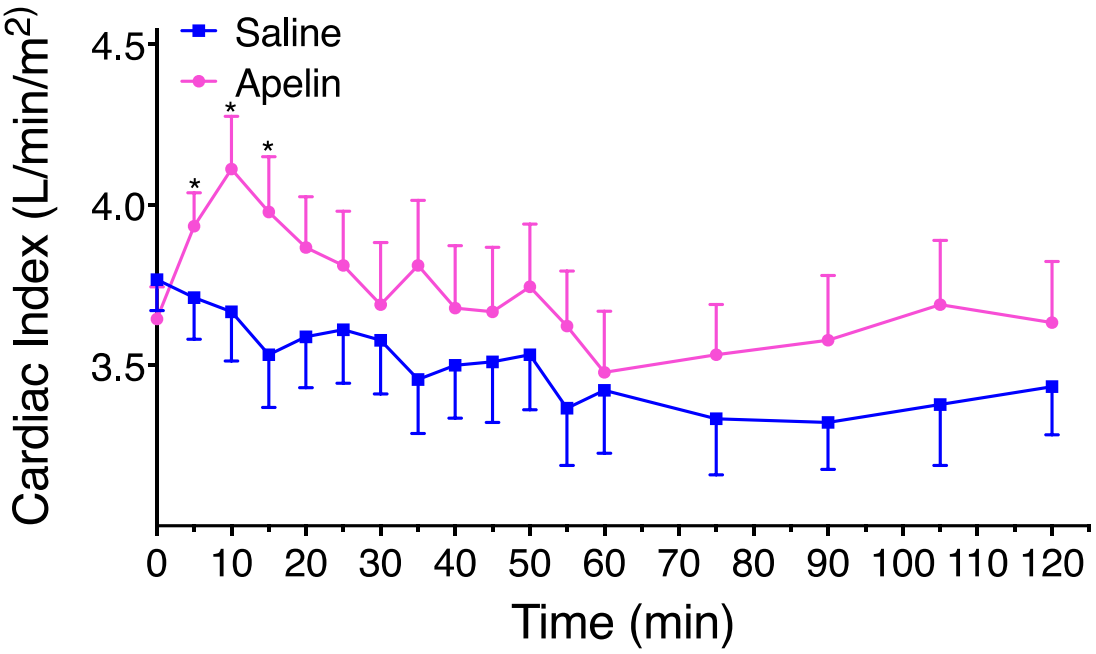


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

The prolonged 2 hour [Pyr[1]]apelin-13 infusion (30 nmol/min) causes significant rise in SVI (omnibus significance P=0.002). Significantly different from saline control $P \leq 0.05$.

In *post-hoc* testing the change is most noticeable within the first 40 minutes at 10, 15, 25, 40 mins timepoints (peaking at the 10 mins timepoint P=0.001), before a gradual return to baseline. * denotes Bonferroni corrected $P<0.003$

Figure 4.4 Effect of two-hour infusion of [Pyr[1]]apelin-13 (30 nmol/min) compared with 0.9 % saline control on cardiac index (CI) in participants with increased BMI.

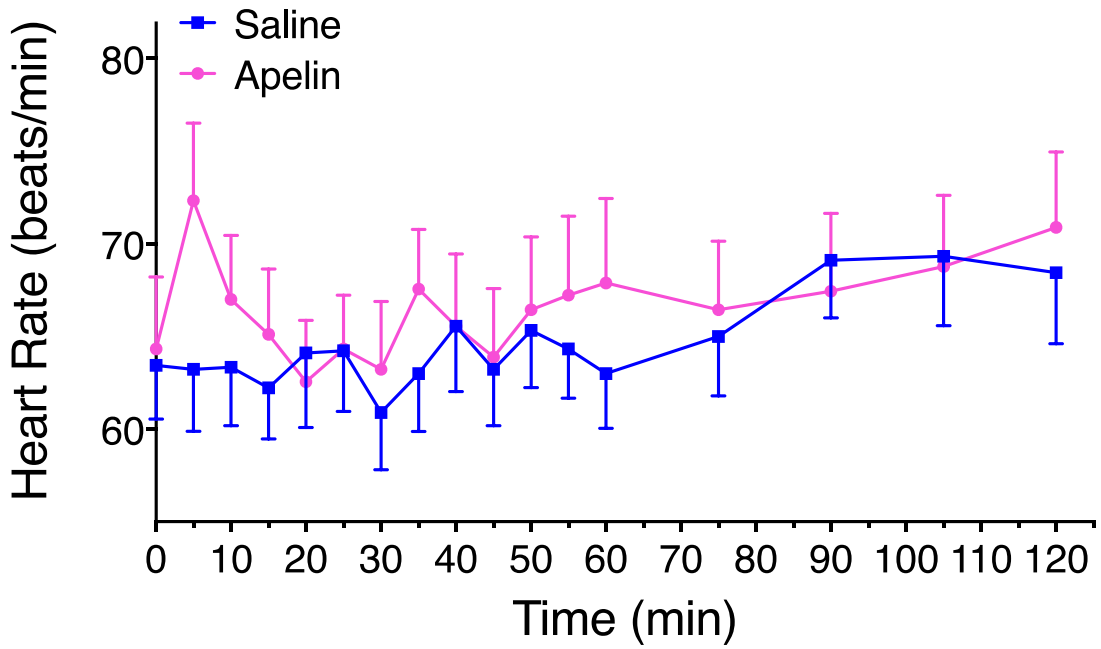


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

The prolonged 2 hour [Pyr[1]]apelin-13 infusion (30 nmol/min) causes significant rise in CI (omnibus significance $P<0.02$). Significantly different from saline control $P\leq0.05$.

In *post-hoc* testing the change is most noticeable within first 15 minutes (peak at 10 mins timepoint $P<0.001$), before a gradual return to baseline. * denotes Bonferroni corrected $P<0.003$

Figure 4.5 Effect of two-hour infusion of [Pyr[1]]apelin-13 (30 nmol/min) compared with 0.9 % saline control on heart rate (HR) in participants with increased BMI

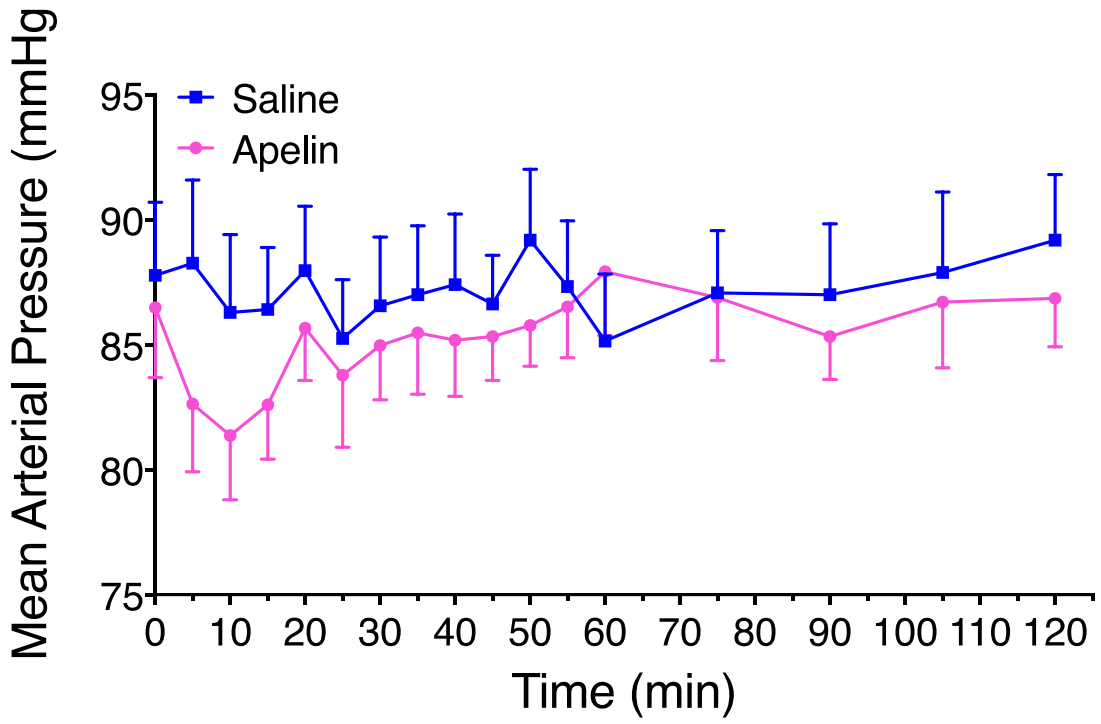


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr[1]]apelin-13 infusion (30 nmol/min) causes significant rise in HR (omnibus significance P=0.047) when compared to the saline control. $P \leq 0.05$ was deemed significant.

No significance was found in post-hoc Bonferroni analyses.

Figure 4.6 Effect of two-hour infusion of [Pyr[1]]apelin-13 (30 nmol/min) compared with 0.9 % saline control on mean arterial pressure (MAP) in participants with increased BMI.

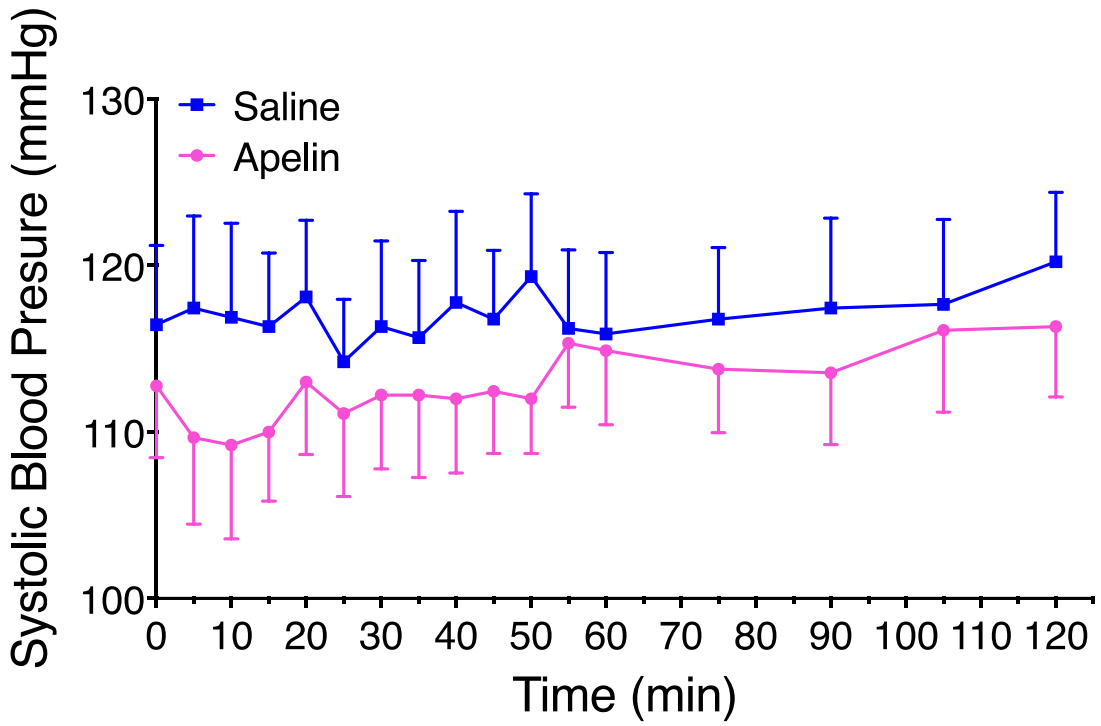


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr[1]]apelin-13 infusion (30 nmol/min) causes a significant drop in MAP (omnibus significance P=0.002). Significantly different from saline control $P \leq 0.05$.

No significance was found in *post-hoc* Bonferroni analyses.

Figure 4.7 Effect of two-hour infusion of [Pyr^{1}]apelin-13 (30 nmol/min) compared with 0.9 % saline control on systolic blood pressure (SBP) in participants with increased BMI.

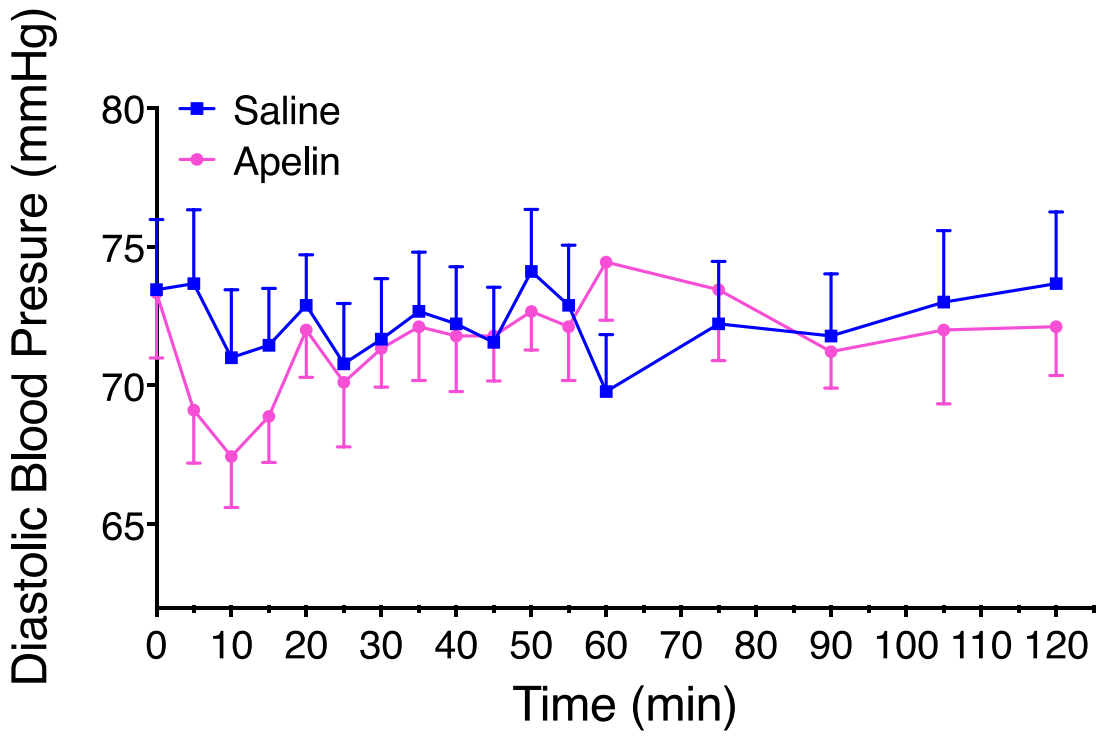


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr^{1}]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr^{1}]apelin-13 infusion (30 nmol/min) does not cause overall significance in SBP (omnibus P=0.104). Significantly different from saline control $P \leq 0.05$.

No significance was found in *post-hoc* Bonferroni analyses.

Figure 4.8 Effect of two-hour infusion of [Pyr[1]]apelin-13 (30 nmol/min) compared with 0.9 % saline control on diastolic blood pressure (DBP) in participants with increased BMI.

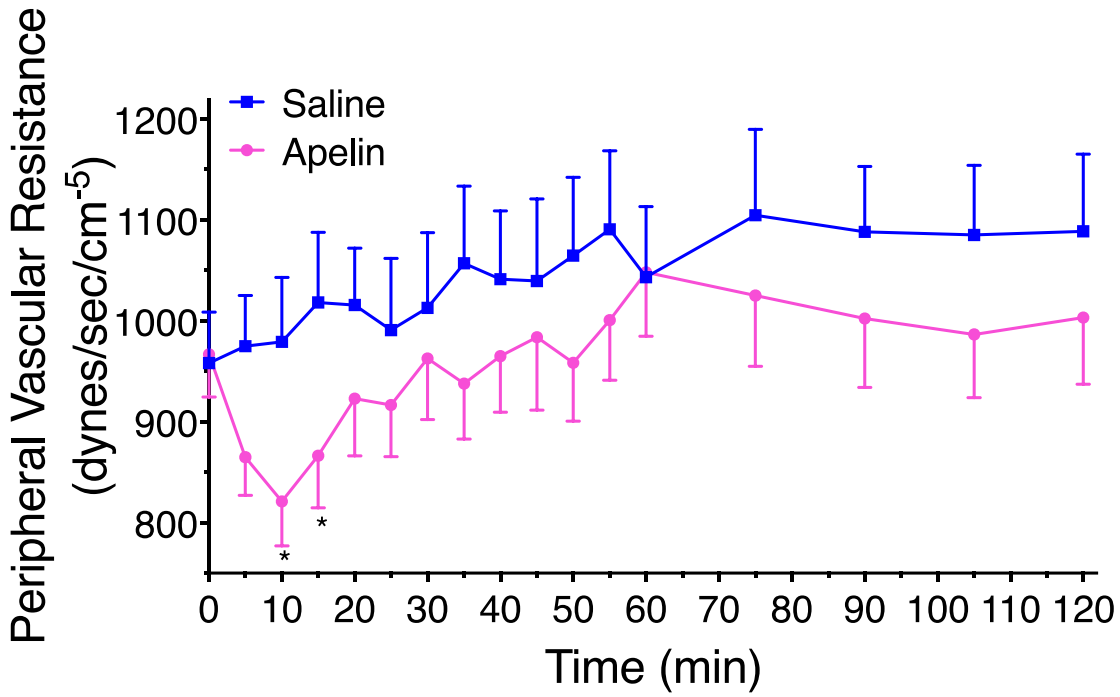


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr[1]]apelin-13 infusion (30 nmol/min) does not cause overall significance in DBP (omnibus P=0.074). Significantly different from saline control $P \leq 0.05$.

No significance was found in *post-hoc* Bonferroni analyses.

Figure 4.9 Effect of two-hour infusion of [Pyr^{1}]apelin-13 (30 nmol/min) compared with 0.9 % saline control on peripheral vascular resistance (PVR) in participants with increased BMI



Measurements were taken at baseline and ar 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr^{1}]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr^{1}]apelin-13 infusion (30 nmol/min) causes significant decline in PVR (omnibus significance P=0.032). Significantly different from saline control $P \leq 0.05$.

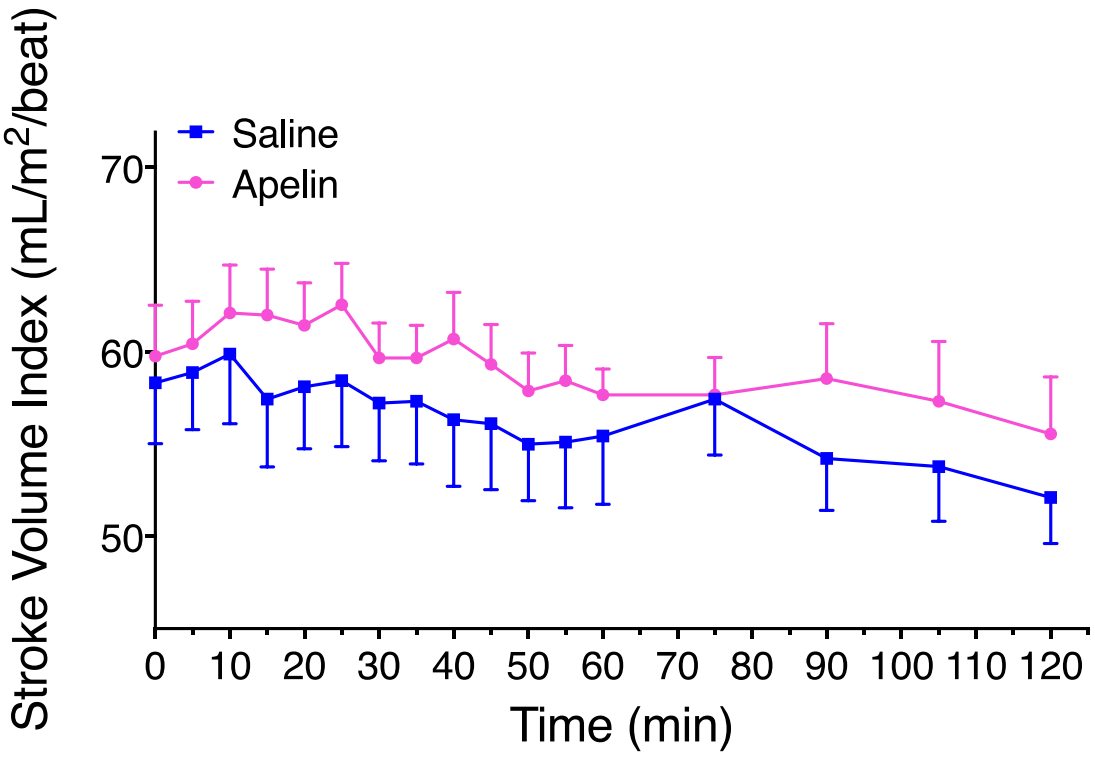
In *post hoc* analyses the change is most noticeable within the first 15 minutes (maximum effect at 10 mins timepoint $P<0.001$), before a gradual return to baseline. * denotes Bonferroni corrected $P<0.003$

4.6.2.1.2 Changes in cardiovascular parameters after two hours of 135 nmol/min [Pyr^{1}]apelin-13 infusion in participants with increased BMI (Group B).

Figures 4.10–4.16 represent changes in cardiovascular variables (SVI, CI, HR, MAP, SBP, DBP, PVR) in nine Study 2 participants (N=9) with increased BMI, following systemic two hours [Pyr^{1}]apelin-13 infusion (135 nmol/min). Variables were measured on the first two dosing visits (V2 and V3) without mixed meal.

There were no recorded overall significant changes in cardiovascular variables (SVI, CI, HR, MAP, SBP, DBP, PVR), tested in ANOVA measurements over the whole infusion period. However, *post hoc* analyses with Bonferroni adjustments showed significant sharp decline at the 5-minute timepoint in PVR (Bonferroni post-test $P<0.003$; Figure 4.16) together with rapid 5-minute increase in both HR and CI (Bonferroni post-test $P<0.002$ and $P<0.001$ respectively; Figure 4.12 and 4.11) with no further change after return to baseline to the end of the study period (120 min).

Figure 4.10 Effect of two-hour infusion of [Pyr^{1}]apelin-13 (135 nmol/min) compared with 0.9 % saline control on stroke volume index (SVI) in participants with increased BMI

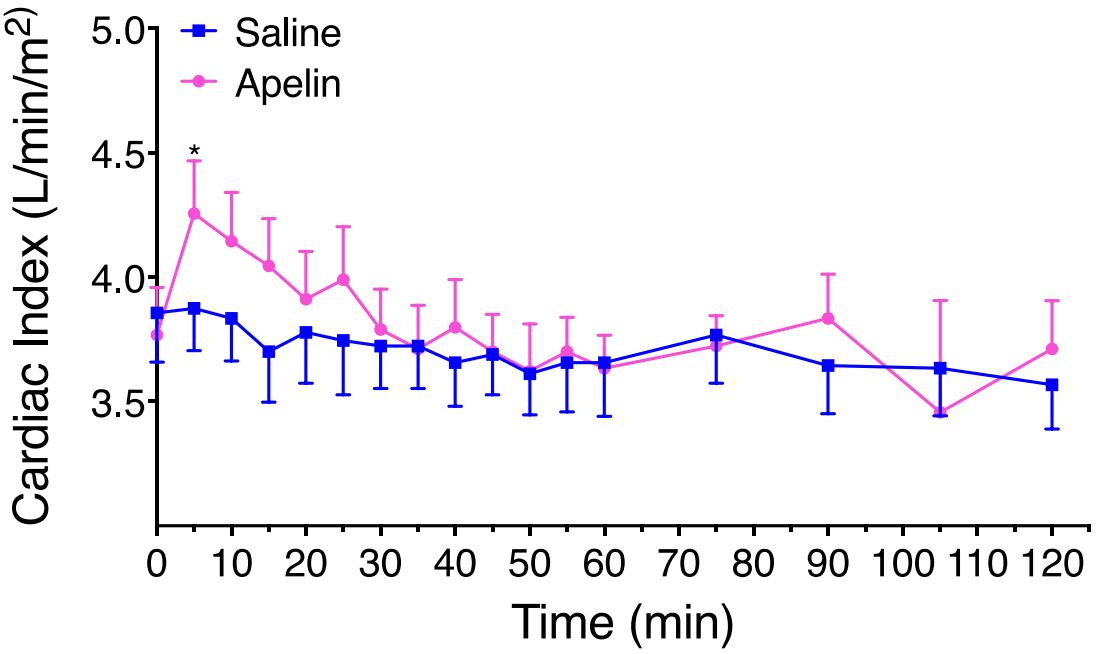


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr^{1}]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr^{1}]apelin-13 infusion (135 nmol/min) does not cause overall significance in SVI (omnibus P=0.958). Significantly different from saline control $P \leq 0.05$.

No significance was found in *post-hoc* Bonferroni analyses.

Figure 4.11 Effect of two-hour infusion of [Pyr[1]]apelin-13 (135 nmol/min) compared with 0.9 % saline control on cardiac index (CI) in participants with increased BMI

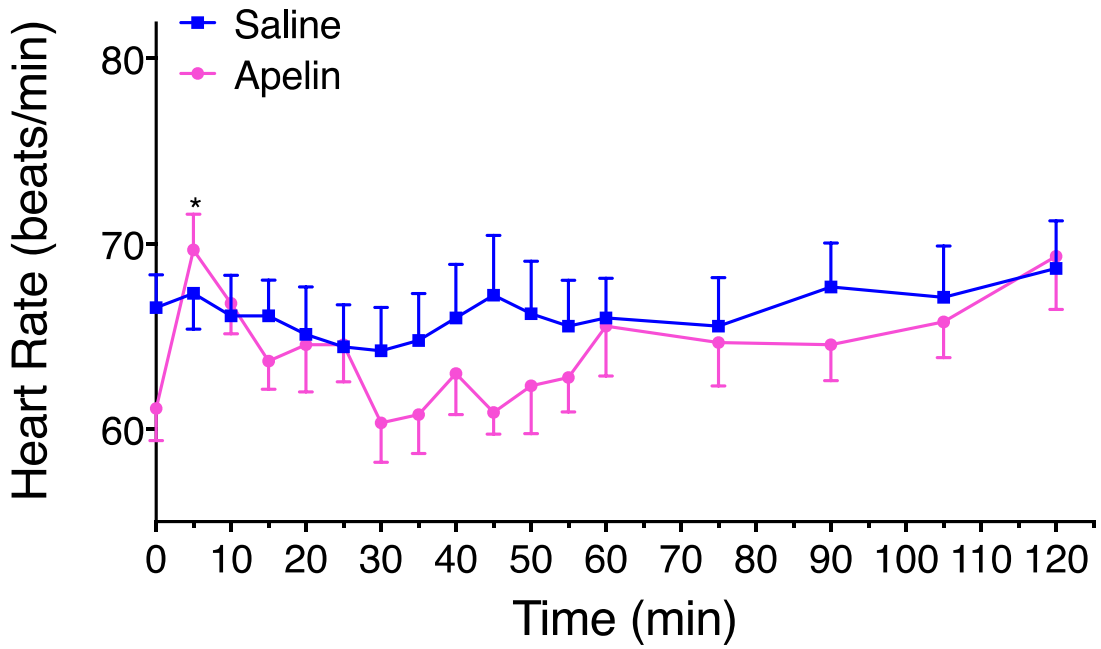


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr[1]]apelin-13 infusion (135nmol/min) does not cause significant overall change (omnibus $P<0.286$). Significantly different from saline control $P\leq0.05$.

In *post-hoc* Bonferroni analysis a significant rise is noticeable within first 5 mins of infusion (peak at 5 mins timepoint $P<0.001$) before a gradual return to baseline. * denotes Bonferroni corrected $P<0.003$.

Figure 4.12 Effect of two-hour infusion of [Pyr[1]]apelin-13 (135 nmol/min) compared with 0.9 % saline control on heart rate (HR) in participants with increased BMI

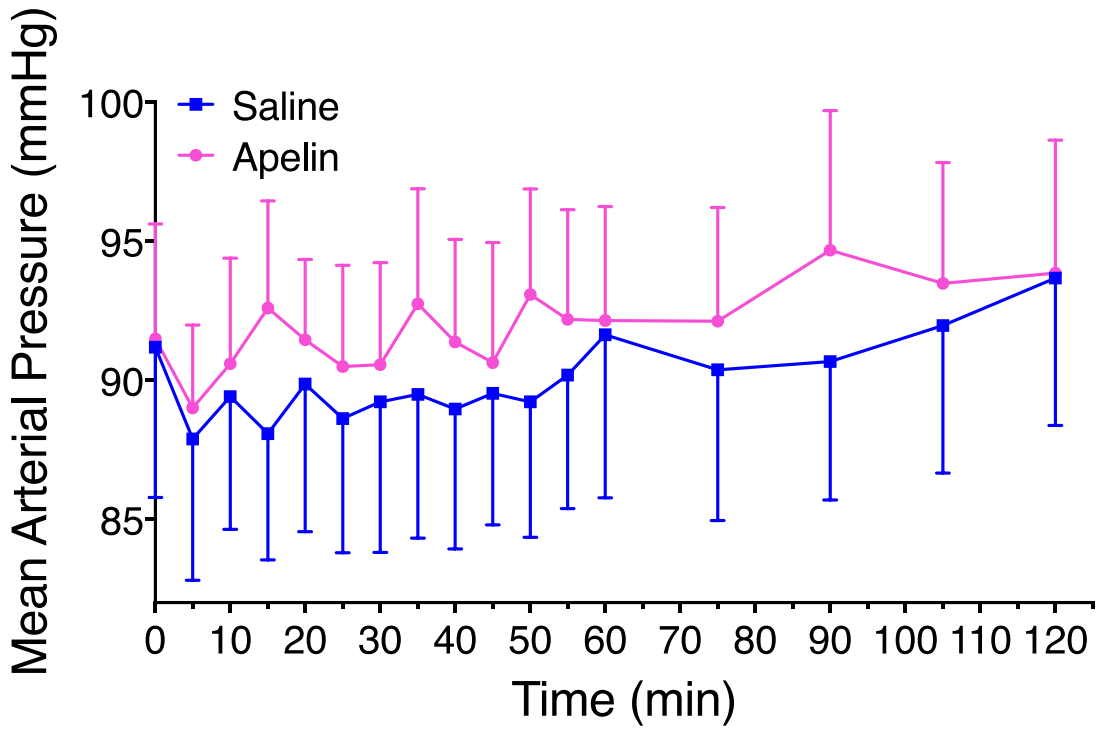


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr[1]]apelin-13 infusion (135 nmol/min) does not cause overall significant change in HR (omnibus P=0.211). Significantly different from saline control $P \leq 0.05$.

In *post-hoc* Bonferroni analysis a significant rise is noticeable within the first 5 mins of infusion (peak at 5 mins timepoint $P<0.002$) before a gradual return to baseline * denotes Bonferroni corrected $P<0.003$.

Figure 4.13 Effect of two-hour infusion of [Pyr[1]]apelin-13 (135 nmol/min) compared with 0.9 % saline control on mean arterial pressure (MAP) in participants with increased BMI

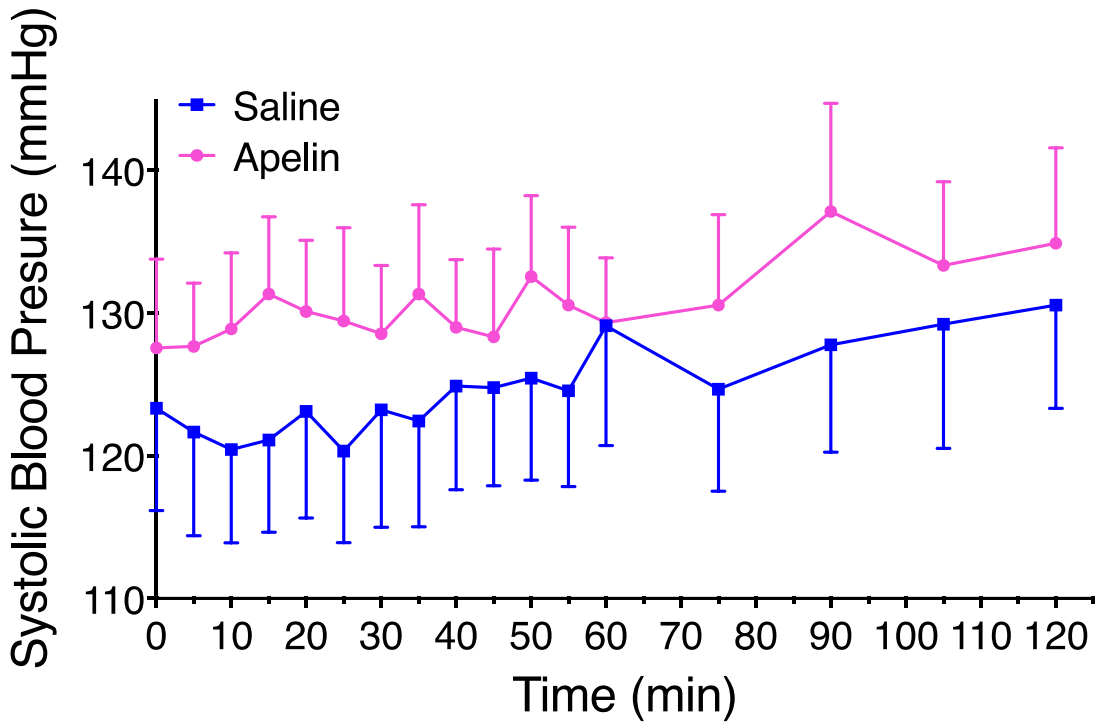


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr[1]]apelin-13 infusion (135 nmol/min) does not cause significant change in MAP (omnibus P=0.901). Significantly different from saline control $P \leq 0.05$.

No significance was found in *post-hoc* Bonferroni analyses.

Figure 4.14 Effect of two-hour infusion of [Pyr^{1}]apelin-13 (135 nmol/min) compared with 0.9 % saline control on systolic blood pressure (SBP) in participants with increased BMI

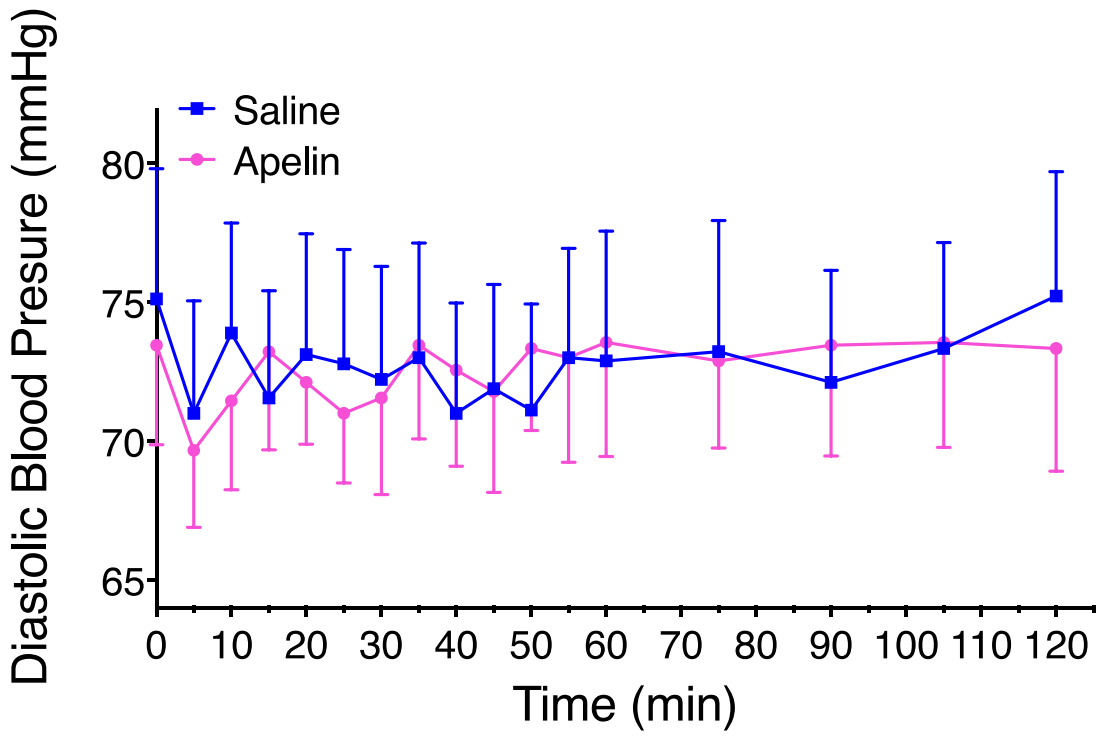


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr^{1}]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr^{1}]apelin-13 infusion (135 nmol/min) does not cause significant change in SBP (omnibus P=0.694). Significantly different from saline control $P \leq 0.05$.

No significance was found in *post-hoc* Bonferroni analyses.

Figure 4.15 Effect of two-hour infusion of [Pyr[1]]apelin-13 (135 nmol/min) compared with 0.9 % saline control on diastolic blood pressure (DBP) in participants with increased BMI

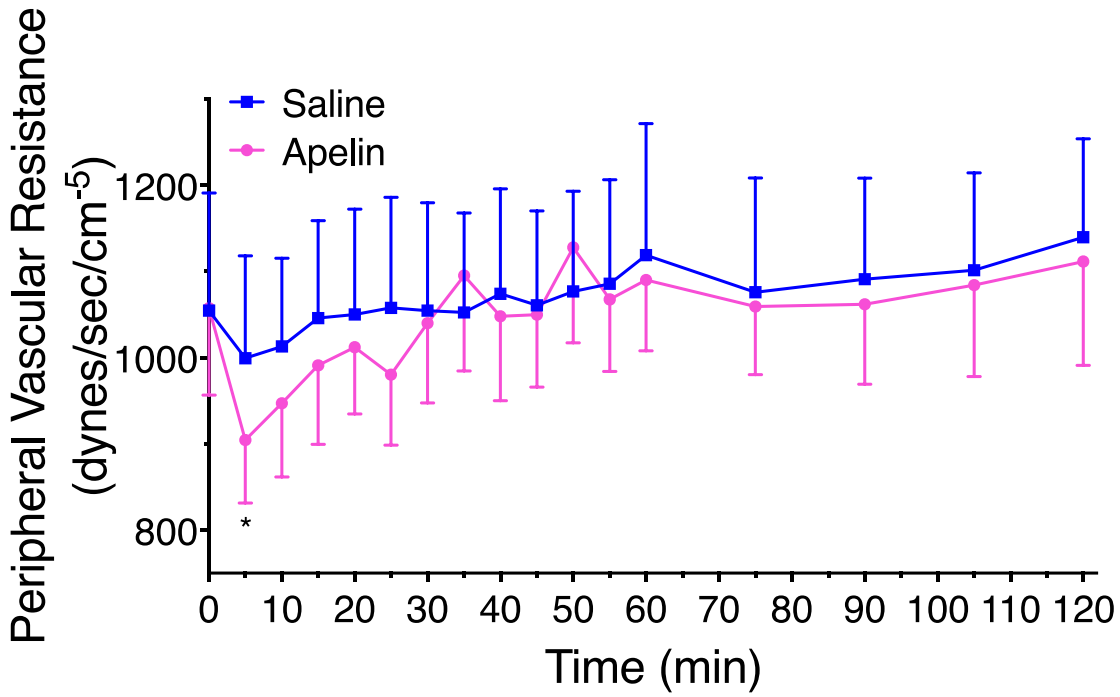


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr[1]]apelin-13 infusion (135 nmol/min) does not cause significant change in DBP (omnibus P=0.897). Significantly different from saline control $P \leq 0.05$.

No significance was found in post-hoc Bonferroni analyses.

Figure 4.16 Effect of two-hour infusion of [Pyr^{1}]apelin-13 (135 nmol/min) compared with 0.9 % saline control on peripheral vascular resistance (PVR) in participants with increased BMI



Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr^{1}]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr^{1}]apelin-13 infusion (135 nmol/min) does not cause significant overall change in PVR (omnibus P=0.394). Significantly different from saline control $P \leq 0.05$.

In *post-hoc* Bonferroni analysis a significant drop is noticeable within first 5 mins of infusion (maximum effects at 5 mins timepoint $P<0.003$) before a gradual return to baseline * denotes Bonferroni corrected $P<0.003$.

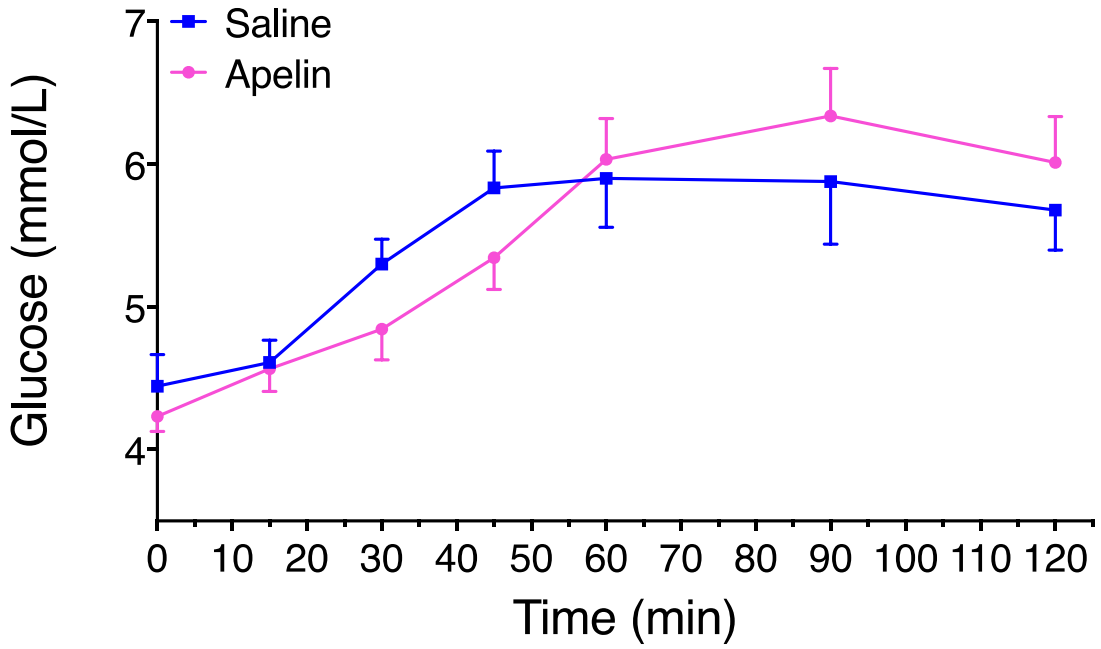
4.6.2.2 Metabolic effects of 2-hour [Pyr^1]apelin-13 infusion in increased BMI participants

4.6.2.2.1 Changes in metabolic parameters after two hours of 30 nmol/min [Pyr^1]apelin-13 infusion in participants with increased BMI (group A).

Figures 4.17–4.19 represent metabolic changes (glucose, insulin and C peptide) in nine Study 2 participants with increased BMI (N=9) following systemic two hours [Pyr^1]apelin-13 infusion in dose 30 nmol/min. Variables are measured during two dosing visits with mixed meal (V4 and V5). Log transformed values of the skewed variables (C peptide and insulin) were used for the 2-way repeated measures ANOVA analyses. Median and interquartile range [IQR] of insulin and C peptide are presented in Table 4.3 to complete data presentation.

[Pyr^1]apelin-13 infusion caused significant reduction in log C peptide values (ANOVA, omnibus *P*=0.027; Figure 4.19) after infusion completion at 120 mins without noted significance in *post-test* Bonferroni's adjustments. There were no overall statistically significant changes in glucose and insulin values (ANOVA, omnibus *P*=0.225 and *P*=0.463 respectively) nor in post-test Bonferroni corrections as shown in Figures 4.17 and 4.18 respectively.

Figure 4.17 Effect of two-hour infusion of [Pyr^{1}]apelin-13 (30 nmol/min) compared with 0.9 % saline control on glucose in participants with increased BMI

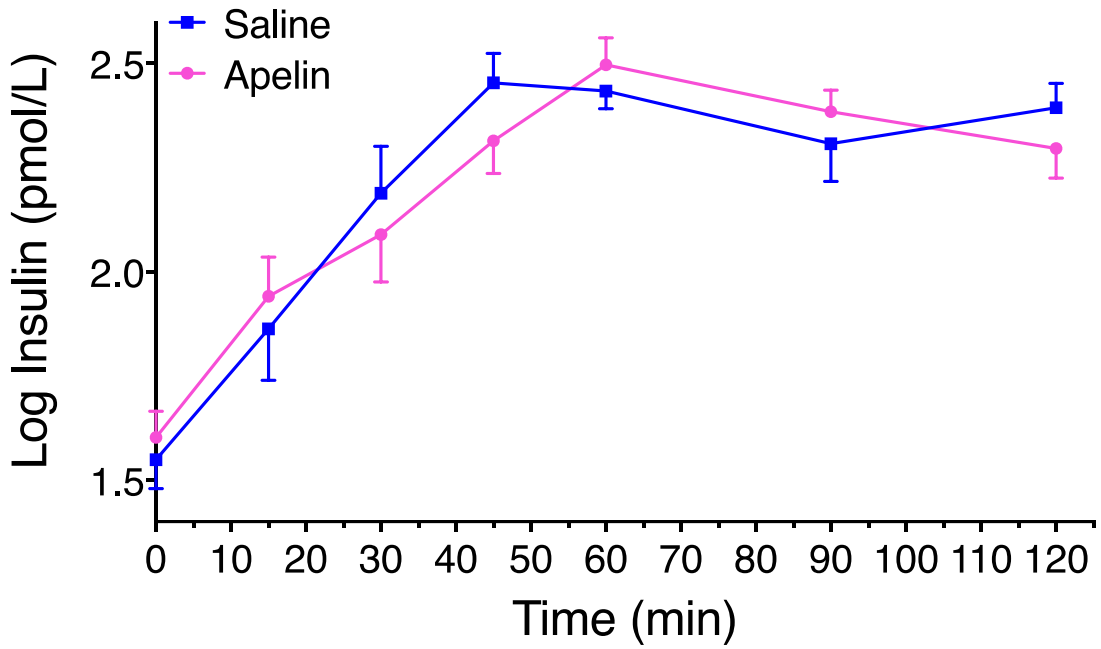


Measurements were taken at baseline followed by 15 minute intervals throughout the first hour and every 30 minutes in the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr^{1}]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr^{1}]apelin-13 infusion (30 nmol/min) does not cause overall significant change in blood glucose (omnibus P=0.225). Significantly different from saline control $P \leq 0.05$.

No significance was found in post-hoc Bonferroni analyses.

Figure 4.18 Effect of two-hour infusion of [Pyr^{1}]apelin-13 (30 nmol/min) compared with 0.9 % saline control on insulin in participants with increased BMI

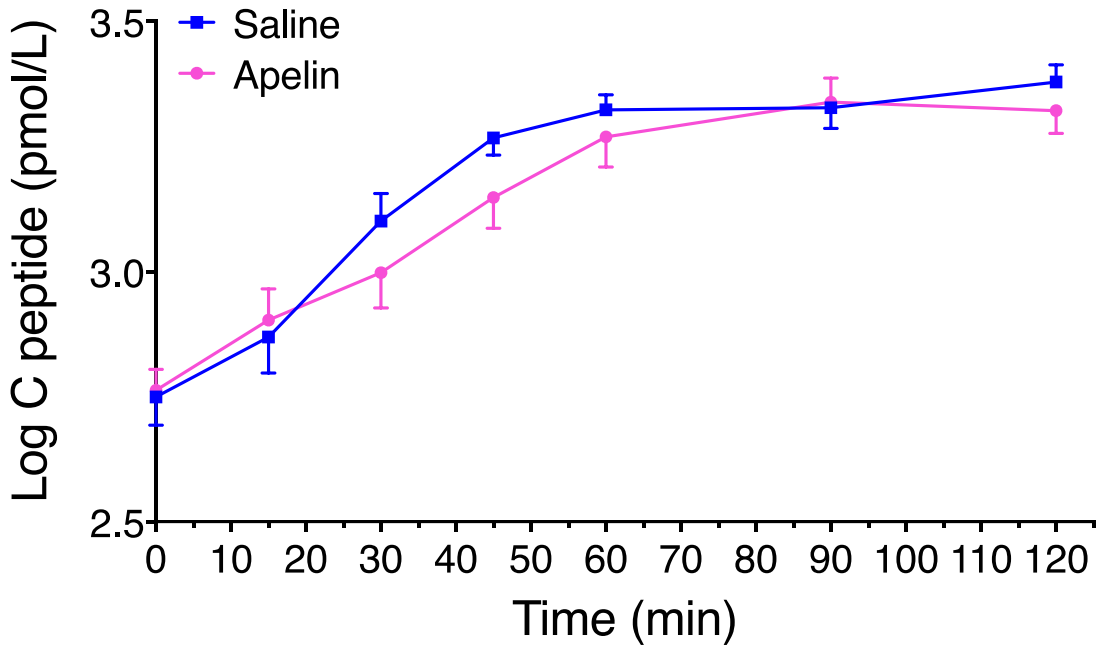


Measurements were taken at baseline followed by 15 minute intervals throughout the first hour and every 30 minutes in the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. Log transformed insulin values were used as variables were skewed. The line chart represents MEAN±SE. The pink line represents [Pyr^{1}]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2-hour [Pyr^{1}]apelin-13 infusion (30 nmol/min) does not cause significant change in insulin (omnibus P=0.463). Significantly different from saline control $P \leq 0.05$.

No significance was found in post-hoc Bonferroni analyses.

Figure 4.19 Effect of two-hour infusion of [Pyr^{1}]apelin-13 (30 nmol/min) compared with 0.9 % saline control on C peptide in participants with increased BMI



Measurements were taken at baseline followed by 15 minute intervals throughout the first hour and every 30 minutes in the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. Log transformed C peptide values were used as variables were skewed. The line chart represents MEAN±SE. The pink line represents [Pyr^{1}]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr^{1}]apelin-13 infusion (30 nmol/min) causes significant decline in C peptide (omnibus P=0.027). Significantly different from saline control $P \leq 0.05$.

No significance was found in *post-hoc* Bonferroni analyses.

Table 4.3 Study 4 dosing visit (V4–5) metabolic parameters at [Pyr[1]]apelin-13 dose 30 nmol/min and normal saline

Treatment	Apelin 30 nmol/L		Normal saline	
Variable (median, [IQR]) / Time/min	Insulin (pmol/L)	C peptide (pmol/L)	Insulin (pmol/L)	C peptide (pmol/L)
0	43 [35-53]	545 [446-696]	34 [25-46]	534 [477-742]
15	64 [49-114]	867 [530-949]	61 [48-111]	787 [579-908]
30	90 [75-221]	996 [698-1200]	177 [96-263]	1220 [923-1560]
45	180 [133-290]	1420 [1040-1560]	272 [220-331]	1770 [1500-2310]
60	322 [180-350]	1950 [1700-2200]	260 [227-322]	2000 [1830-2400]
75	-	-	-	-
90	263 [176-313]	2300 [1730-2440]	193 [144-271]	1930 [1840-2620]
105	-	-	-	-
120	207 [147-270]	2490 [1520-2610]	223 [214-300]	2200 [1970-2680]

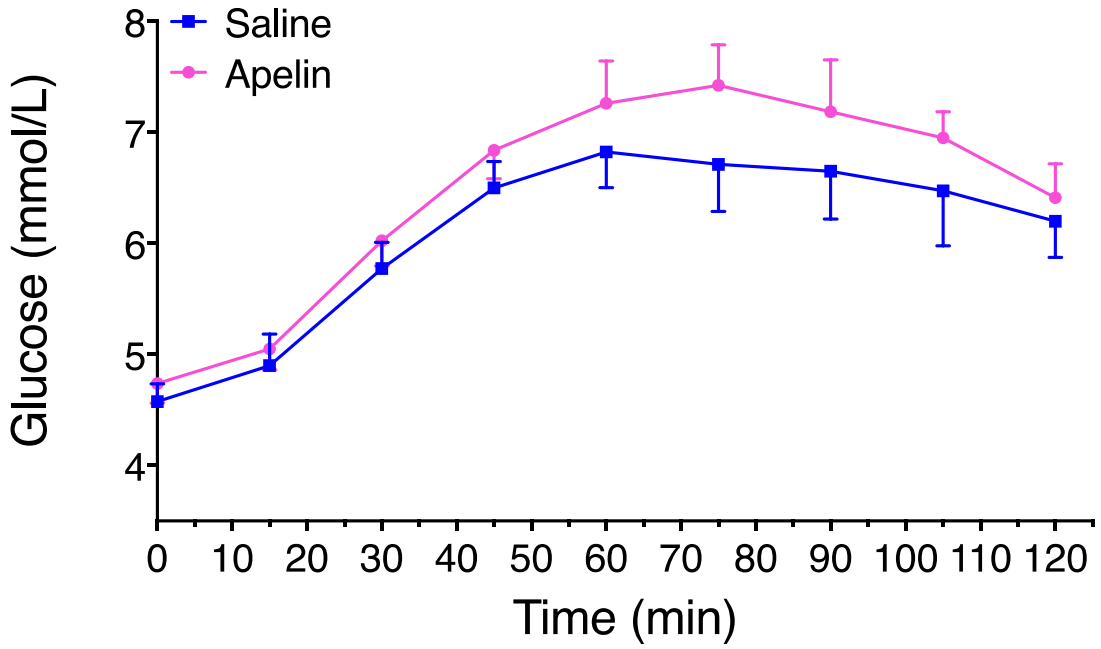
At dosing visit V4-V5 each participant received a 2 hour infusion of [Pyr[1]]apelin-13 at 30 nmol/min or normal saline (control). Insulin and C peptide data are not normally distributed and are presented as median and interquartile range [IQR].

4.6.2.2.2 Changes in metabolic parameters after two hours of 135 nmol/min [Pyr^{1}]apelin-13 infusion in participants with increased BMI (Group B).

Figures 4.20–4.22 represent metabolic changes (glucose, insulin and C peptide) in eight Study 2 participants with increased BMI (N=8) following systemic two hour [Pyr^{1}]apelin-13 infusion in dose 135 nmol/min. Variables were measured on dosing visits with mixed meal. Log transformed values of the skewed variables (C peptide and insulin) were used for the 2-way repeated measures ANOVA analyses. Median and interquartile range [IQR] of insulin and C peptide are presented in Table 4.4 to complete data presentation.

There were no overall statistically significant changes for any of the tested metabolic variables; glucose, log insulin and log C peptide (ANOVA, omnibus P=0.648; P=0.170; P=0.190 respectively) nor in post-test Bonferroni corrections as shown in Figures 4.20 to 4.22 respectively.

Figure 4.20 Effect of two-hour infusion of [Pyr^{1}]apelin-13 (135 nmol/min) compared with 0.9 % saline control on glucose in participants with increased BMI

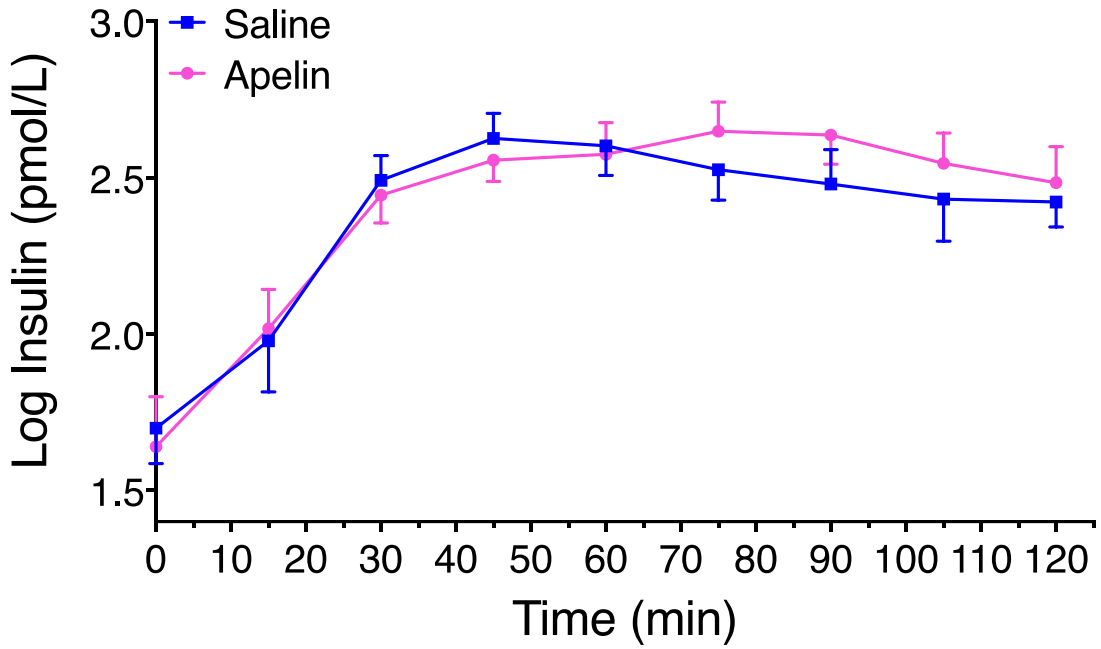


Measurements were taken at baseline followed by 15 minute intervals throughout the 2 hour infusion. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr^{1}]apelin-13 and the blue line normal saline. Number of participants is N=8.

Prolonged 2 hour [Pyr^{1}]apelin-13 infusion (135 nmol/min) does not cause significant change in blood glucose (omnibus P=0.648). Significantly different from saline control $P \leq 0.05$.

No significance was found in *post-hoc* Bonferroni analyses.

Figure 4.21 Effect of two-hour infusion of [Pyr[1]]apelin-13 (135 nmol/min) compared with 0.9 % saline control on insulin in participants with increased BMI

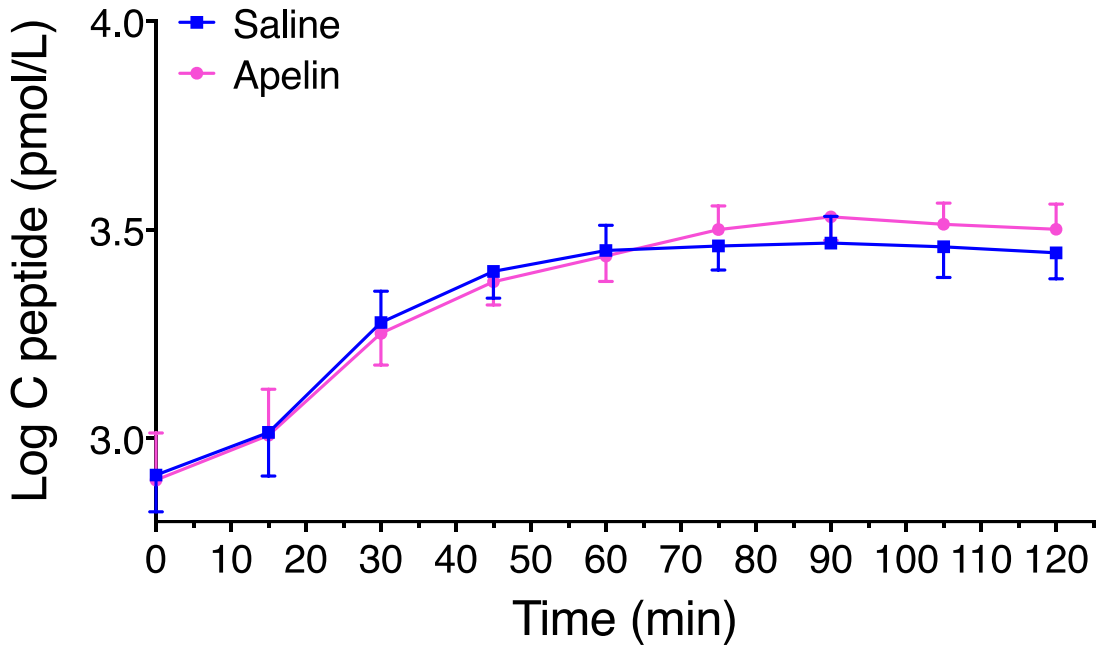


Measurements were taken at baseline followed by 15 minute intervals throughout the 2 hour infusion. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. Log transformed insulin values were used as variables were skewed. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=8.

Prolonged 2-hour [Pyr[1]]apelin-13 infusion (135 nmol/min) does not cause significant change in insulin (omnibus P=0.170). Significantly different from saline control $P \leq 0.05$.

No significance was found in *post-hoc* Bonferroni analyses.

Figure 4.22 Effect of two-hour infusion of [Pyr^{1}]apelin-13 (135 nmol/min) compared with 0.9 % saline control on C peptide in participants with increased BMI



Measurements were taken at baseline followed by 15 minute intervals throughout the 2 hour infusion. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. Log transformed C peptide values were used as variables were skewed. The line chart represents MEAN±SE. The pink line represents [Pyr^{1}]apelin-13 and the blue line normal saline. Number of participants is N=8.

Prolonged 2 hour [Pyr^{1}]apelin-13 infusion (135 nmol/min) does not cause significant changes in C peptide values (omnibus P=0.190). Significantly different from saline control $P \leq 0.05$.

No significance was found in *post-hoc* Bonferroni analyses.

Table 4.4 Study 4 dosing visit (V4-5) metabolic parameters at [Pyr[1]]apelin-13 dose 135 nmol/min and normal saline

Treatment	Apelin 30 nmol/L		Normal saline	
Variable (median, [IQR]) / Time/min	Insulin (pmol/L)	C peptide (pmol/L)	Insulin (pmol/L)	C peptide (pmol/L)
0	51 [25-116]	849 [685-1370]	50 [38-89]	842 [665-1277]
15	108 [68-206]	1105 [874-1685]	107 [58-210]	1235 [941-1375]
30	291 [182-420]	1965 [1347-2720]	414 [226-448]	2290 [1665-2562]
45	346 [271-453]	2615 [2042-2932]	489 [318-599]	2755 [2217-3532]
60	407 [307-468]	3025 [2442-3260]	435 [328-516]	3245 [2505-3557]
75	448 [377-490]	3270 [2977-3380]	356 [233-443]	2955 [2410-3457]
90	389 [346-464]	3410 [2795-3717]	323 [223-409]	2895 [2457-3407]
105	353 [235-403]	3335 [2712-3565]	306 [250-406]	2785 [2527-4132]
120	285 [181-355]	3195 [2422-3437]	253 [184-373]	2570 [2305-3462]

At dosing visit V4-V5 each participant received a 2 hour infusion of [Pyr[1]]apelin-13 at 135 nmol/min or normal saline (control). Insulin and C peptide data are not normally distributed and are presented as median and interquartile range [IQR].

4.6.3 Safety and tolerability study results

All participants during [Pyr[1]]apelin-13 infusion remained haemodynamically stable without pathological ECG or cardiac telemetry changes. Adverse events (AEs) participants experienced during study involvement are shown in Table 4.5.

Table 4.5 Tolerability of [Pyr[1]]apelin-13 infusions at doses 30 and 135 nmol/min in participants with increased BMI

Adverse event severity	Apelin dose	Adverse event	Causality	Events N=9
Severe	135 nmol/min	hepatitis A	not related	1
Moderate	135 nmol/min	migraine	not related	1
Mild	30 nmol/min	bruise on cannulation site	definitely related	2
		skin efflorescence	not related	1
		common cold	not related	1
	135 nmol/min	nasal congestion	probably related	1
		facial flushing	probably related	1
		large bowel movement day after visit	unlikely related	1

One serologically confirmed case of viral hepatitis A with full medical recovery was reported as a serious adverse event (SAE) at [Pyr[1]]apelin-13 dose 135 nmol/min. Although not being related to the [Pyr[1]]apelin-13 infusion, the event led to participant mid-study exclusion after completing V2 and V3. Two mild AEs presented as nasal congestion and facial flushing were possibly related to a higher 135 nmol/min [Pyr[1]]apelin-13 dose, and have fully resolved within minutes of infusion discontinuation.

4.7 Discussion

Study 2 was a “proof of concept” study which provided for the first-time demonstration of beneficial cardiovascular and metabolic effects of apelin, infused in isolation to participants with increased body mass index (BMI) who served as a clinical model of insulin resistance.

This Study has been designed to detect and to compare clinical effects of two [Pyr^{1}]apelin-13 doses (Participants Group A and B receiving 30 and 135 nmol/min respectively) on insulin sensitivity and cardiovascular actions. Study results were aimed to guide [Pyr^{1}]apelin-13 dose selection tested in Study 3, rather than to illuminate underlying cardiovascular and metabolic molecular mechanisms.

4.7.1 Safety and tolerability at [Pyr^{1}]apelin-13 dose 30 and 135 nmol/min

This Study continued testing safety and tolerability at a lower (30 nmol/min) but also higher apelin dose (135nm/min) than acutely administered in previous clinical studies (Barnes *et al.*, 2013, Gourdy *et al.,* 2018). We monitored symptoms, vital parameters (HR, BP) and cardiac telemetry recordings and have not found any concerns related to [Pyr^{1}]apelin-13 safety and tolerability over continuous 2-hour systemic infusion. Except for twice reported subjective feeling of nasal congestion and facial flushing, likely underlined with [Pyr^{1}]apelin-13 induced vasodilation effect as addressed in Chapter 3 discussion (Section 3.8), participants have not reported AEs in causal relation to [Pyr^{1}]apelin-13 infusion.

4.7.2 Cardiovascular and metabolic effects of [Pyr^{1}]apelin at dose 30 nmol/min

A short-term increase in CVI and SVI, and a reduction in PVR and MAP was recorded with the lower dose of 30 nmol/min [Pyr^{1}]apelin-13 in participants with increased BMI (Group A), who were otherwise healthy and have not been taking any relevant concomitant medication.

Although pre-clinical research confirmed apelin to be a potent inotrope (Szokodi *et al.,* 2002; Berry *et al.*, 2004; Ashley *et al.,* 2005; Maguire *et al.,* 2009), our study has not been designed to determine whether a noted increase in CI (magnitude around 0.5 L/min/m^{2}) can be partially attributed to known positive *in vitro* and *in-vivo* inotropic actions or is it simply a secondary response to peripheral vasodilation (Lee *et al.,* 2000; Tatemoto *et al.,* 2001; Cheng *et al.,* 2003; Maguire *et al.,* 2009).

Heart rate transiently increased within the first five minutes of the infusion, as would be typical for a vasodilating agent and has previously been demonstrated in healthy participants and HF patients (Cheng *et al.,* 2003; Japp *et al,* 2010; Barnes *et al.,* 2013).

A short-term reduction in MAP (magnitude around 5–7 mmHg) has been noted and [Pyr^{1}]apelin-13 (30 nmol/min) caused a small but significant vasodilation within the first 15 minutes, in agreement with previously reported studies in both healthy volunteers and HF patients (Japp *et al.*, 2010). In addition, these CV results are in agreement with those reported by Barnes *et al* (2013) where [Pyr^{1}]apelin-13 in systemic prolonged 6-hours infusion at dose 30 nmol/min increased CI, lowered MAP and PVR in HF patients (New York Heart Association; NYHA class II to III). Those patients were on concurrent treatment with β-blockers, ACE inhibitors or AT_1 antagonists, which is indicating that [Pyr^{1}]apelin-13 mediated these actions even under angiotensin and adrenergic receptor blockade.

The molecular mechanism is thought to occur via binding of [Pyr^{1}]apelin-13 to receptors expressed on vascular endothelium resulting in the release of vasodilators. Although the magnitude of the response is comparatively small, [Pyr^{1}]apelin-13 induced vasodilation may be beneficial in the states of insulin resistance. The fact that insulin has a role not only as a hormone essential in maintaining euglycemia, but also in maintaining blood flow and tissue perfusion (Lalande et Romero, 2019) has become well recognised. Apelin, by increasing insulin sensitivity in insulin resistant subjects (Gourdy *et al.,* 2018), could therefore be enhancing the role of insulin in stimulating micro-vascular perfusion, blood flow of skeletal muscle and subcutaneous adipose tissue. Others have shown that apelin improves insulin-stimulated, endothelium-dependent and independent vasodilatory responsiveness whilst reducing both Ang II and ET-1 induced vasoconstriction, suggesting that apelin agonists could beneficially improve haemodynamic abnormalities in obesity-associated IR (Schinzari *et al.,* 2017).

During the course of the present study, Gourdy and colleagues (2018) reported key data from a randomized, double-blind, placebo-controlled, cross-over study in eight healthy overweight men who underwent hyperinsulinaemic-euglycaemic clamping procedure. They showed that [Pyr^{1}]apelin-13 at a systemic dose of 30 nmol/kg (administered at constant rate of 250 pmol/kg/min) significantly improved insulin sensitivity during two hours of peptide administration. These results provided first evidence in humans for the beneficial insulin-sensitising action of [Pyr^{1}]apelin-13, previously observed only pre-clinically in insulin clamping experiments in mice (Dray *et al.,* 2008).

Although clinical study by Gourdy *et al.* provided proof of concept of apelin being an insulin-sensitizer in overweight participants with increased insulin resistance, the effect of acute intravenous apelin perfusion during a clamp was tested alongside insulin. In contrast, the present study was designed to detect the actions of [Pyr^{1}]apelin-13 in isolation in our selected groups of participants, and to mimic normal eating behaviour with a meal tolerance test.

We compared the postprandial metabolic effects of continuous two hour [Pyr^{1}]apelin infusion and placebo in participants with increased BMI. Our objective in measuring changes over this length of time was to maximise the ability to measure any modulatory action of [Pyr^{1}]apelin-13 on the postprandial peaks of insulin and blood glucose after the meal challenge. C-peptide and insulin are released from the pancreas at the same time and in about equal amounts but C-peptide has a longer half-life than insulin and is more stable and reliable for laboratory assessment.

In the increased BMI participants receiving [Pyr^{1}]apelin-13 dose 30 nmol/min, we recorded a significant drop in C peptide over the time of infusion during the mixed meal challenge. The significant reduction in C peptide measured in our participants with increased BMI was in agreement with findings made by Gourdy and colleagues (Gourdy *et al.,* 2018) indicating apelin infusion increased insulin sensitivity. However, these data more closely represents usual physiological processes since we measured the effects of [Pyr^{1}]apelin-13 after a mixed meal.

4.7.3 Cardiovascular and metabolic effects of [Pyr^{1}]apelin at dose 135 nmol/min

While [Pyr^{1}]apelin at dose 135 nmol/min was well tolerated, as indicated previously, we did not find overall significant metabolic nor cardiovascular change over 2 hours infusion period. However, interestingly *post hoc* analyses revealed significant sharp PVR decline and rapid HR and CI increase at the 5-minute time point, before coming back to the baseline.

As both A and B participant groups receiving [Pyr^{1}]apelin-13 dose 30 and 135 nmol/min respectively, were similar in clinical characteristics it is unlikely that a lack of response could be explained by the inter-group clinical differences (Table 4.1). In addition, biological [Pyr^{1}]apelin-13 activity was confirmed in β-arrestin functional assays (Nyimanu *et al.,* 2019a) and we have previously shown using mass spectrometry that infusion achieved the predicted concentration of [Pyr^{1}]apelin-13 in the plasma (Nyimanu *et al.,* 2019b).

Therefore, the lack of observed effect is unlikely to be caused by metabolic degradation of the peptide and we propose that the most likely explanation for lack of a dose response is the agonist induced internalisation and desensitization of the receptor at the higher dose (Pope. *et al.,* 2016).

This receptor desensitisation effects should be overcome by the use of biased agonists in the chronic therapeutic setting (Yang *et al.*, 2015; Brame *et al.*, 2015; Read *et al.*, 2021)

4.8 Study limitations

We acknowledge some limitations with our approach. In the absence of any previous metabolic studies, power calculations were based on published data for the detection of significant changes in cardiovascular parameters.

Study participants were selected as they presented the model of insulin resistance. However, some of $HOMA_{IR}$ calculations could not have been performed as some of the insulin samples were partially haemolysed which affected the credibility of the result. Despite that, based on their clinical phenotype participants were presumed to be insulin resistant (Freeman et Pennings, 2021).

4.9 Study conclusion

In conclusion, in participants with increased BMI in response to the [Pyr^1]apelin-13 infusion (30 nmol/min), we noted significant changes in C-peptide levels, indirectly indicating an increase in insulin sensitivity and confirmed beneficial cardiovascular effects of vasodilation and subsequent increase of cardiac index.

Based on these tolerability, cardiovascular and metabolic data we are going ahead using [Pyr^1]apelin-13 at dose 30 mol/min in the subsequent Study 3 to test [Pyr^1]apelin-13 CV and metabolic effects in T2DM patients.

Chapter 5:

Study 3 – First in man study investigating cardiovascular and metabolic effects of prolonged infusion of [Pyr^{1}]apelin-13 in patients with Type 2 diabetes mellitus

5.1 Background

Obesity is predisposing individuals to the development of IR and subsequently T2DM (Section 1.2). Interest in the connection of apelin and T2DM has been established ever since apelin has been identified as an adipose tissue secreted adipokine hormone (Boucher *et al.*, 2005). and has ~~especially~~ grown after beneficial dual metabolic and cardiovascular effects of apelin have been confirmed in animals and more importantly non-diabetic humans (Section 1.3.2.2.1 and 1.3.2.2.2.1). In addition to apelin being a potential insulin sensitizer exerting positive inotropic and vasodilating actions, several human observational studies have highlighted apelin as a promising biomarker for detection of heart failure (Goidescu *et al.,* 2021) and impaired glucose homeostasis (Section 1.3.2.2.2.2.2)

Metabolic and cardiovascular diseases present major health challenges of the modern world, alone and in combination, resulting in a heightened interest in the development of drugs with dual cardiovascular and metabolic actions. The incidence of HF is higher in diabetic patients compared to the non-diabetic population (Jia *et al*., 2018) and CV disease contribute significantly to T2DM associated mortality and morbidity (Gaede *et al.,* 2003). Despite existing therapies, there remains scope for the development of new therapeutic approaches via novel pathways distinct from the current standard of care, that could be either used alone or in combination with current drugs. Already in clinical use some diabetes targeting agents such as glucagon-like peptide 1 (GLP-1) agonists and gliflozins (sodium-glucose transport protein 2 – SGLT2) inhibitors have been additionally proven to have concomitant beneficial cardiovascular effects. Targeting apelin/APJ signalling pathways presents an interesting therapeutic approach as [Pyr^{1}]apelin-13 showed insulin sensitising properties in animals and overweight humans (Dray *et al.,* 2008; Gourdy *et al.*, 2018) and ameliorated diabetic cardiomyopathy in animals (Section 1.3.3.1).

Despite a great body of encouraging cardiometabolic preclinical and clinical data, the effects of the apelin/APJ system on diabetes complications remain unclear. In animal models, apelin seems to improve diabetes induced kidney hypertrophia and normalises obesity-associated cardiac hypertrophy, but negatively promotes retinal angiogenesis in diabetic retinopathy (Section 1.3.3).

However, as human *in vivo* studies testing effects of apelin have not yet been conducted in T2DM patients, definitive conclusions regarding cardiometabolic effects and impact on diabetes complications remain elusive.

In Study 2 conducted in participants with increased BMI systemic prolonged [Pyr[1]]apelin-13 administration at dose 30 nmol/min decreased C peptide (P=0.027), increased CI (*P*<0.02) and lowered MAP (*P*=0.002) and PVR (*P*=0.032) without serious adverse events. Based on the Study 2 findings we progressed into research involving T2DM patients, and now are presenting a first in human study investigating the cardiovascular and metabolic pharmacological properties of [Pyr[1]]apelin-13 during systemic infusions in T2DM patients.

[Pyr[1]]apelin-13 dose 30 nmol/min was guided by results in participants with increased BMI serving as a model of insulin resistance, which made them similar to T2DM patients. This dose was also consistent with the effective dose used in previously published work (Japp *et al*., 2010; Barnes *et al.,* 2013)

5.2 Study aims, objectives and endpoints

5.2.1 Study aims

Study 3 aims were to estimate safety and tolerability, cardiovascular and metabolic effects of 2-hour [Pyr[1]]apelin-13 infusion at dose 30 nmol/min in patients with Type 2 diabetes mellitus

5.2.2 Study objectives

Study objective was to define a range of safe doses of intravenous [Pyr[1]]apelin-13 which may have an effect on glucose homeostasis and cardiovascular physiology.

5.2.3 Study endpoints

Study endpoints after infusion of [Pyr[1]]apelin-13 were to detect:

1. Changes in parameters evaluating safety and tolerability including BP, HR, POC glucose, ECG, cardiac telemetry
2. Changes in markers of glucose homeostasis including glucose, C-peptide, insulin
3. Changes in parameters of cardiovascular haemodynamics including cardiac index measured by bioreactance method

5.3 Study hypothesis

We tested for the first time systemic cardiovascular effects and effect on insulin sensitivity of [Pyr^{1}]apelin-13 at dose 30 nmol/min in patients with Type 2 diabetes mellitus with the following hypothesis:

1. intravenously infused [Pyr^{1}]apelin-13 will be safe and tolerable
2. intravenously infused [Pyr^{1}]apelin-13 will increase cardiac index (CI)
3. intravenously infused [Pyr^{1}]apelin-13 will increase insulin sensitivity

These hypotheses were tested with systemic intravenous infusions of the [Pyr^{1}]apelin-13 inducing cardiac and metabolic effects monitored as described in Section 4.3.

5.4 Methods

Study 3 was a randomized double-blind study in patients with Type 2 diabetes mellitus. In total thirteen patients with T2DM underwent screening and nine patients met the study inclusion criteria to complete four study dosing visits.

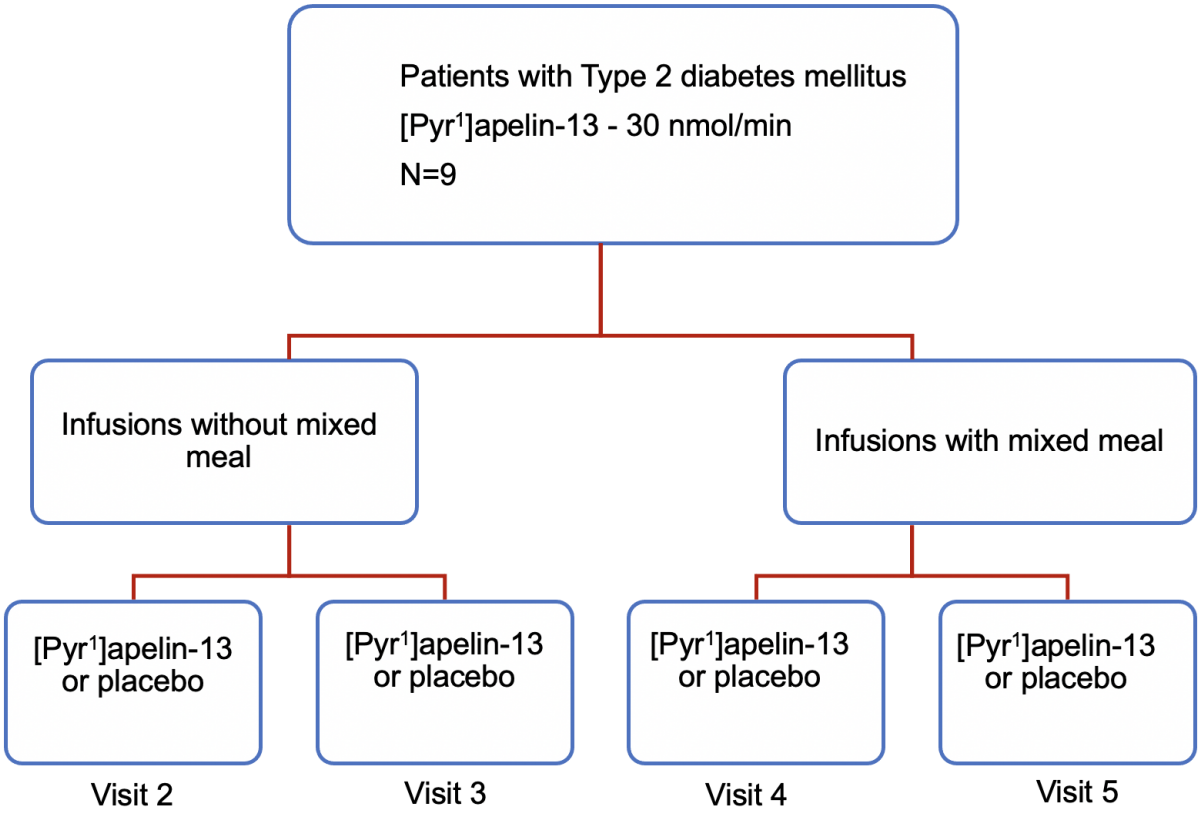
Patients study inclusion criteria:

- Documented diagnosis of T2DM
- Treated with oral antihyperglycemic therapy
- BMI measured 18–34.9 kg/m^2
- Current non-smoker
- Age range 18–75 years

Exclusion criteria were previously listed in Section 2.2.2.2.

Participants attended study visits as is shown in Figure 5.1. All participants attended dosing visits at morning time, whilst previously fasted for 12 hours and withdrew their diabetes medications until the dosing study visit was completed. Each participant was given lunch after the infusion. Measurements at predefined times during infusion were shown in Figure 4.2 and outlined in Sections 2.4.3.7 and 2.4.3.2.

Figure 5.1 Protocol for Study 3



Nine Type 2 diabetes mellitus patients were infused with [Pyr[1]]apelin-13 at dose 30 nmol/min. Each participant was used as their own control, attending four double blind dosing visits with [Pyr[1]]apelin-13 or normal saline placebo administered in random order. Visit 2 and 3 were conducted without mixed meal (for measurement of cardiovascular parameters) and Visit 4 and 5 with mixed meal challenge (for measurement of post-prandial metabolic parameters). Mixed meal challenge is described in Section 2.4.3.5

5.5 Data analyses

The sample size calculation was described in Section 2.7.1.3. Data distribution was tested using Q-Q plots and Shapiro-Wilk test.

Demographic and baseline cardiovascular and metabolic participants' data are given as means±SD and analysed using Excel software (Microsoft office, IBM). Unpaired 2-tailed Student's *t*-tests were used to compare differences between the Study 3 and Study 2

participants, as both groups received same [Pyr[1]]apelin-13 infusion dose 30 nmol/min. A probability of $P<0.05$ was considered significant.

N values are the number of participants who underwent study dosing visits. Cardiovascular and metabolic variables were reported for analyses of study endpoints in assessing the effects of [Pyr[1]]apelin-13 infusions. Cardiovascular variables; CI, SVI, HR, MAP and PVR were analyzed in the first two dosing visits without mixed meal challenge (V2 and V3). Metabolic variables; glucose, insulin and C peptide were reported and analyzed in dosing visits with preceded mixed meal challenge (V4 and V5). Data are given as means±SE. For the skewed variables (insulin and C peptide) log-transformed values were used for the analyses. The significance was determined by 2-way ANOVA repeated measures (SPSS version 26; IBM, New York, USA), with *post hoc* testing with Bonferroni's adjustments. Data are graphically presented with Graphpad Prism (GraphPad Software Inc, USA) with help of Dr Janet Maguire. Greenhouse-Geisser corrected *P*-values were used if Mauchly's test revealed a violation of sphericity.

5.6 Study 3 results

5.6.1 Subjects characteristics

Table 5.1 displays baseline demographic, biochemical, cardiovascular and clinical characteristics of nine T2DM patients and comparisons to Study 2 Group A participants. A comparison is displayed as Study 2 Group A participants served as a model of IR and received the same [Pyr[1]]apelin-13 dose at 30 nmol/min.

Data were collected in altogether nine T2DM patients. Patients have been predominantly white (N=7) in both compared groups, however T2DM patients were significantly older in mean age (60.3±7.8 *versus* 29.3±10.6 years; $P<0.001$). There was no significant difference in mean BMI which is ranging in overweight and class I obese category for both groups. However, despite comparable BMI, T2DM participants showed more trend toward abdominal fat distribution based on mean waist circumference measurements (102.7±14.2 *versus* 89.3±11 cm; $P=0.04$), with significantly less mean body fat percentage (27.4±7.9 *versus* 35.7±6 %; $P=0.02$).

Eight T2DM patients had previous smoking history with median smoking exposure of 10.1 pack-years. Mean diabetes disease duration was 10.1±8.1 years and diabetes control was suboptimal with average Hba1c of 58.3±14 mmol/mol. The group has been shown to be insulin resistant by mean $HOMA_{IR}$ ranging 3.1±1.8.

One patient was solely treated with a diabetes diet. Eight other patients were treated with oral diabetes medications which included: metformin therapy (N=7, daily dose range 500–2000 mg), gliclazide (N=2, daily dose range 40–160 mg), dapagliflozin (N=1, dose 10 mg) and sitagliptin (N=1, dose 100 mg). Most patients were treated with monotherapy (five taking metformin and one gliclazide), one with dual therapy (metformin and sitagliptin combination) and one with triple therapy (metformin, gliclazide, dapagliflozin combination).

Three patients were diagnosed with diabetic neuropathy, one with diabetic neuropathy and nephropathy, one with diabetic neuropathy and coronary artery disease and one with diabetic retinopathy. Three patients were free of diabetes complications.

Four patients were treated for hypertension. One patient (N=1) was on dual treatment with combination of calcium channel blocker amlodipine and AT_1 – receptor blocker candesartan. Others were on monotreatment with AT_1 – receptor blocker losartan (N=1) and ACE-inhibitor lisinopril (N=2) with average suboptimal control of arterial hypertension.

One T2DM patient had no lipid blood disorder whilst others had raised blood lipids, with (N=5) or without treatment (N=3). Out of five patients treated for dyslipidaemia; one patient (N=1) was on dual statin and fibrate treatment (pravastatin and fenofibrate) whilst others (N=4) were on statin treatment (atorvastatin). Only two out of five treated patients were well controlled with lipids in aimed reference range.

The results of haemodynamic measurements show there was no significant difference in HR between groups. T2DM patients had lower mean CI, with borderline significance between groups (3.4±0.4 *versus* 3.8±0.3 L/min/m^2; *P*=0.05). Mean MAP and PVR were significantly higher in T2DM than in Group A Study 2 participants (98.6±9.7 *versus* 87.8±8.7 mmHg; *P*=0.02 and 1133.2±205.5 *versus* 958.3±151.1 dynes/sec/cm^5; *P*=0.05 respectively). All baseline cardiovascular measurements in both groups remained within normal reference range.

Patients had no other relevant comorbidities to the study.

Table 5.1 Inter-group comparison of demographic, biochemical and baseline cardiovascular variables in T2DM patients and Group A Study 2 participants infused with 30 nmol/min [Pyr^{1}]apelin-13 dose

Study 3	Study 3 – T2DM patients N=9	Study 2 – Group A N=9	*P*
Age (years)	60.3±7.8 [50–73]	29.3±10.6 [19–56]	<0.001
Gender, M/F	8 M, 1 F	3M, 6 F	
Ethnicity, W/B/H	7 W, 1 B, 1 H	7 W, 1 B, 1 H	
BMI (kg/m^2)	29.7±2.9 [26–34.6]	29.8±2.1 [26.6–32.7]	0.94
Waist circumference (cm)	102.7±14.2 [86–125]	89.3±11 [68–103]	0.04
Body fat mass (%)	27.4±7.9 [9.4–38]	35.7±6 [29–44.3]	0.02
Ex-smokers, n	8	1	
Smoking (pack-years)	10.1±5.5	0.05	
Baseline biochemistry			
Hba$_{1c}$ (mmol/mol)	58.3±14 [33–87]	34±3.1 [29–38]	<0.001
fasting glucose (mmol/L)	7.1±1.7 [4–8.8]	-	
fasting insulin (pmol/L)	61.8±34.9 [14–123]	-	
HOMA$_{IR}$	3.1±1.8 [0.9–5.9]	-	
T2DM duration (years)	10.1±8.1 [2–25]	-	
Total cholesterol (mmol/L)	3.9±0.6 [2.8–4.6]	-	
HDL cholesterol (mmol/L)	1.2±0.3 [0.68–1.53]	-	
LDL cholesterol (mmol/L)	2.1±0.5 [1.3–2.9]	-	
Triglycerides (mmol/L)	1.3±0.6 [0.7–2.5]	-	

Study 3	Study 3 – T2DM patients N=9	Study 2 – Group A N=9	*P*
Medication, n			
ACE/ARB	3	-	
β-blocker	-	-	
Ca channel blocker	1	-	
statins	5	1	
diabetes diet	9	-	
metformin	7	-	
sitagliptin	1	-	
gliclazide	2	-	
dapagliflozin	1	-	
Baseline cardiovascular			
heart rate (bpm)	62.4±8.6 [50–78]	63.4±8.7 [50–77]	0.8
MAP (mm Hg)	98.6±9.7 [80.66–112]	87.8±8.7 [77–105.3]	0.02
PVR (dynes/sec/cm^{-5})	1133.2±205.5 [896.3–1539.62]	958.3±151.1 [768.2–1186.7]	0.05
CI (L/min/m^2)	3.4±0.4 [2.7–3.9]	3.8±0.3 [3.4–4.1]	0.05

Gender; M – Male, F – Female. Ethnicity; W – White, B – Black, A – Asian, H – Hispanic. ACE – Angiotensin – Converting Enzyme Inhibitors. ARB – Angiotensin II Receptor Blockers. BMI – Body Mass Index. HR – Heart Rate. MAP – Mean Arterial Pressure. PVR – Peripheral Vascular Resistance. CI – Cardiac Index. Hba1c – Glycated Haemoglobin. $HOMA_{IR}$ – Homeostatic Model Assessment for Insulin Resistance. Values are represented as means±SD and [range]. Significance was determined by unpaired 2-tailed Student's *t*-tests. *P*-value <0.05 was deemed significant.

5.6.2 Cardiovascular effects of 2-hour [Pyr[1]]apelin-13 infusion in T2DM patients

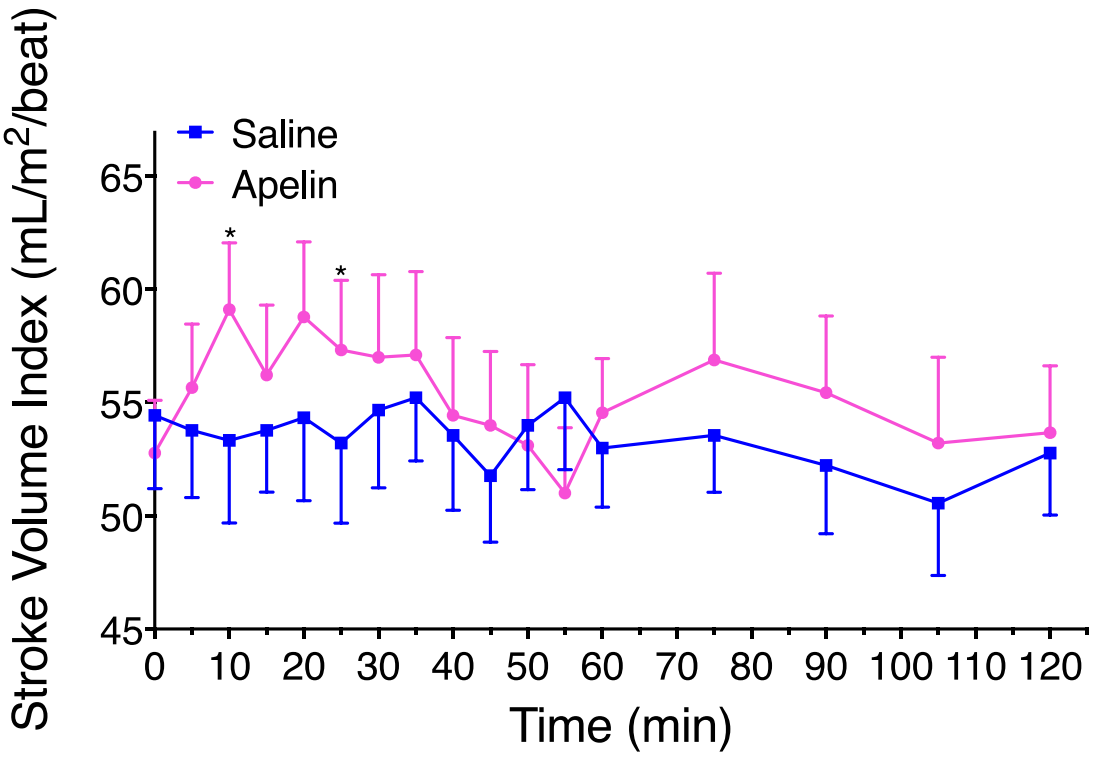
Figures 5.2–5.8 represent cardiovascular changes (SVI, CI, HR, MAP, SBP, DBP, PVR) in nine (N=9) T2DM patients following systemic two hour [Pyr[1]]apelin-13 infusion (30 nmol/min) measured on first two dosing visits (V2 and V3) without mixed meal.

Continuous infusion of intravenous [Pyr[1]]apelin-13 increased SVI over 2 hours, with most noticeable changes within the first 25 minutes, before their gradual return to baseline (ANOVA, omnibus P=0.05; Bonferroni post-test peak P=0.002 at 10 min; Figure 5.2). Cardiac index was overall significantly increased with post-hoc significant changes noted within first 10 mins (ANOVA omnibus P<0.001; Bonferroni post-test peak P<0.001 at 10 mins; Figure 5.3).

[Pyr[1]]apelin-13 infusion caused overall significant decline in PVR (ANOVA omnibus significance P=0.01; Figure 5.8) with most notable changes within first 15 minutes, but not reaching significance in post-test analyses. There were no overall or Bonferroni post-test significant changes in HR (ANOVA omnibus P=0.082; Figure 5.4) and MAP measurements (ANOVA omnibus P=0.541; Figure 5.5). There were no overall significant changes in systolic and diastolic blood pressure measurement as shown in Figures 5.6 and 5.7 respectively.

In summary, saline infusion placebo had no effect on cardiovascular variables. [Pyr[1]]apelin-13 infusion overall significantly increased CI (ANOVA, P<0.001; Figure 5.3) and SVI (ANOVA, P=0.05; Figure 5.2). Also, it caused a decline in PVR (ANOVA, P=0.01; Figure 5.8), without effect on MAP (ANOVA, P=0.541; Figure 5.5) and HR (ANOVA, P=0.082; Figure 5.4). *Post-hoc* testing with Bonferroni adjustments revealed CV changes in CI and SVI were most noticeable after 10 minutes before a gradual return to baseline after 25 min as shown in Figures 5.2–5.8.

Figure 5.2 Effect of two-hour infusion of [Pyr[1]]apelin-13 (30 nmol/min) compared with 0.9 % saline control on stroke volume index (SVI) in T2DM patients

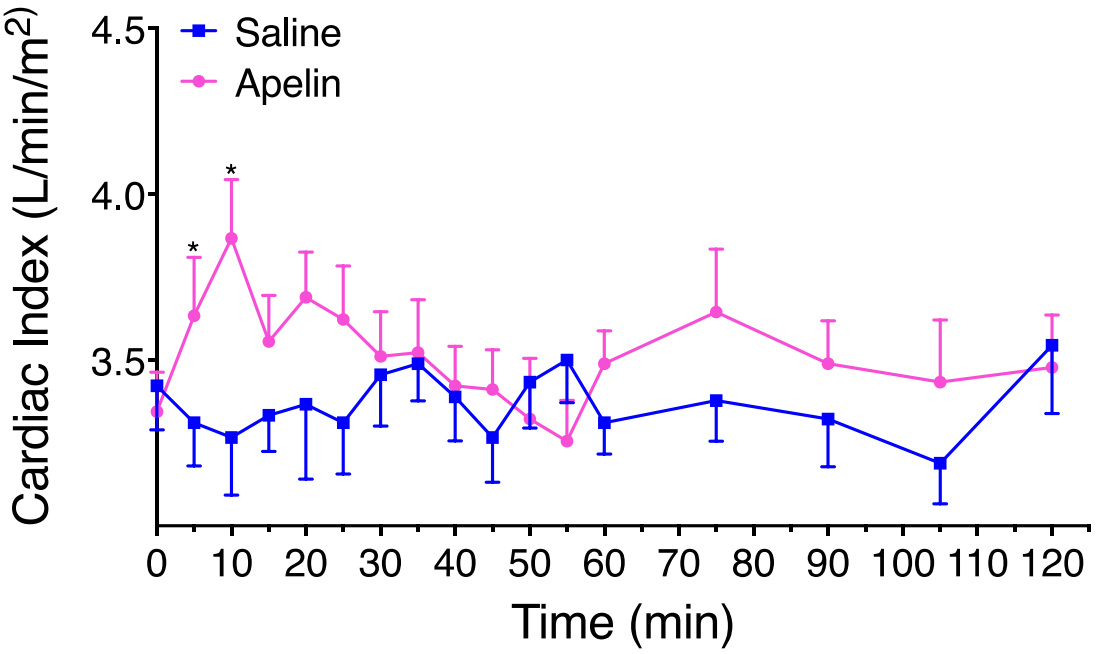


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr[1]]apelin-13 infusion (30 nmol/min) causes significant rise in SVI (omnibus significance P=0.05). Significantly different from saline control $P \leq 0.05$.

In post-hoc testing the change is most noticeable within the first 25 minutes at 10 and 25 minute timepoints (peak at 10 minutes timepoint P=0.002), before a gradual return to baseline.
* denotes Bonferroni corrected $P<0.003$

Figure 5.3 Effect of two-hour infusion of [Pyr[1]]apelin-13 (30 nmol/min) compared with 0.9 % saline control on cardiac index (CI) in T2DM patients

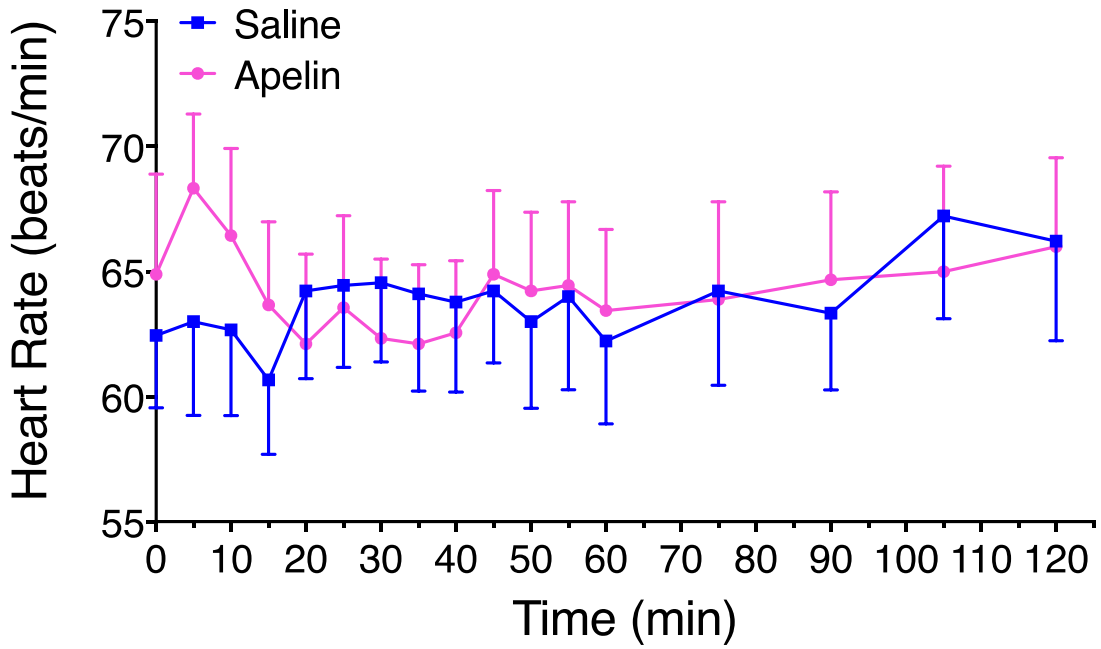


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals in the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr[1]]apelin-13 infusion (30 nmol/min) causes significant rise in CI (omnibus significance $P<0.001$). Significantly different from saline control $P\leq0.05$.

In *post-hoc* analyses the change is most noticeable within the first 10 minutes at 5 and 10 minute timepoints (peak at 10 mins timepoint $P<0.001$), before gradual return to baseline. * denotes Bonferroni corrected $P<0.003$

Figure 5.4 Effect of two-hour infusion of [Pyr[1]]apelin-13 (30 nmol/min) compared with 0.9 % saline control on heart rate (HR) in T2DM patients

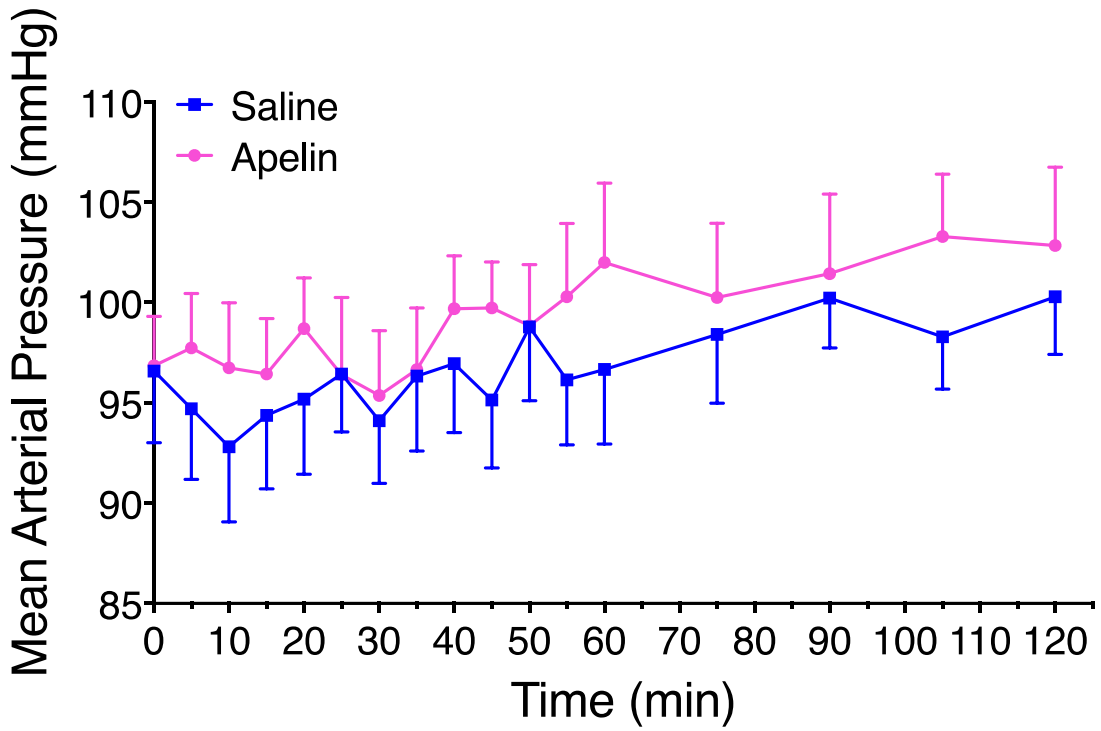


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals during the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr[1]]apelin-13 infusion (30 nmol/min) does not cause significant change in HR (omnibus significance P=0.082). Significantly different from saline control $P \leq 0.05$.

No significance was found in post-hoc Bonferroni analyses.

Figure 5.5 Effect of two-hour infusion of [Pyr[1]]apelin-13 (30 nmol/min) compared with 0.9 % saline control on mean arterial pressure (MAP) in T2DM patients

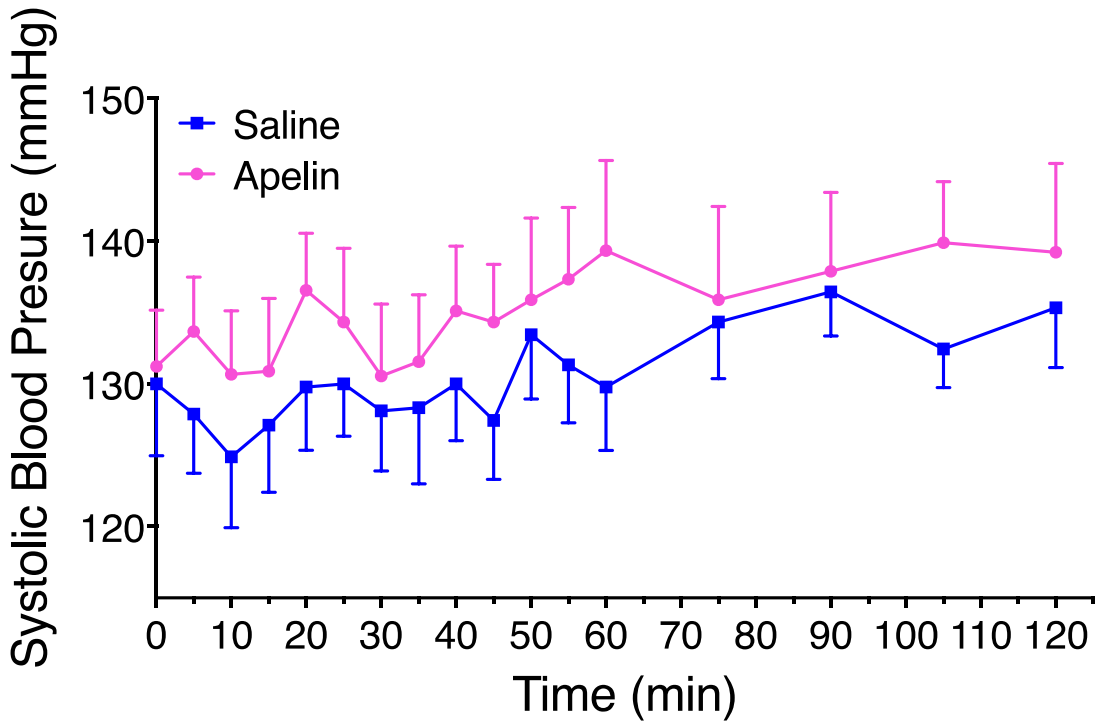


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals in the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr[1]]apelin-13 infusion (30 nmol/min) does not cause significant change in MAP (omnibus significance P=0.541). Significantly different from saline control $P \leq 0.05$.

No significance was noted in post-hoc analyses with Bonferroni adjustments.

Figure 5.6 Effect of two-hour infusion of [Pyr^{1}]apelin-13 (30 nmol/min) compared with 0.9 % saline control on systolic blood pressure (SBP) in T2DM patients

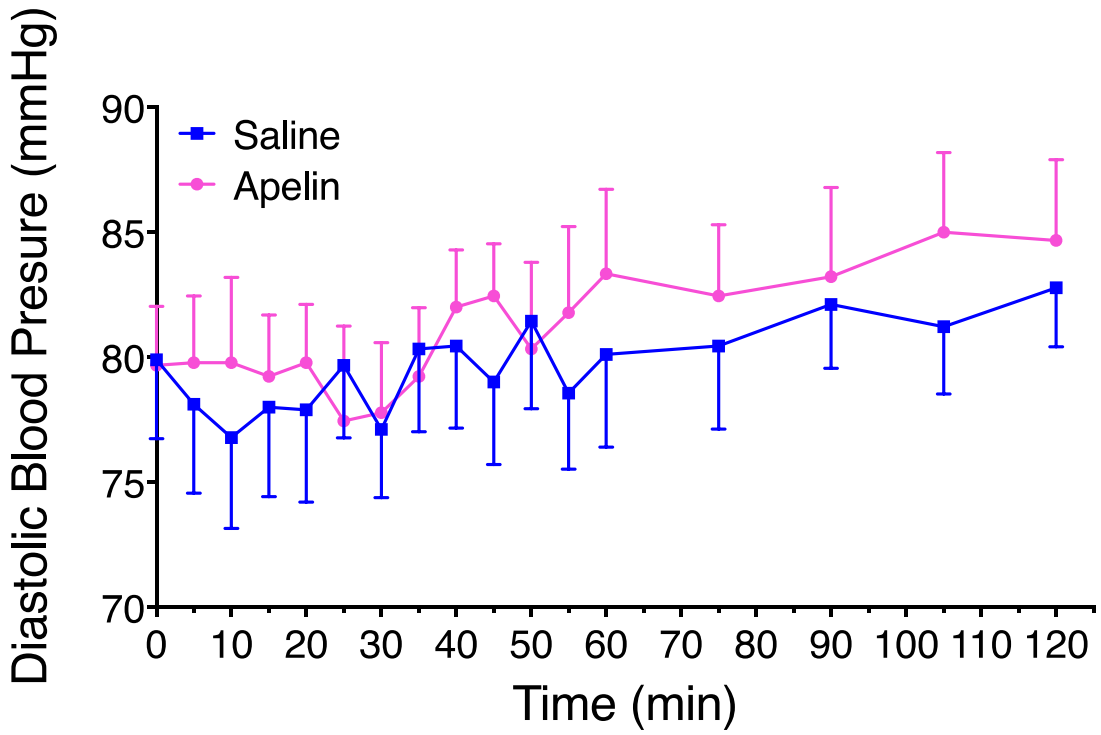


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals in the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr^{1}]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr^{1}]apelin-13 infusion (30 nmol/min) does not cause significant change in SBP (omnibus significance P=0.442). Significantly different from saline control $P \leq 0.05$.

No significance was noted in post-hoc analyses with Bonferroni adjustments.

Figure 5.7 Effect of two-hour infusion of [Pyr^{1}]apelin-13 (30 nmol/min) compared with 0.9 % saline control on diastolic blood pressure (DBP) in T2DM patients

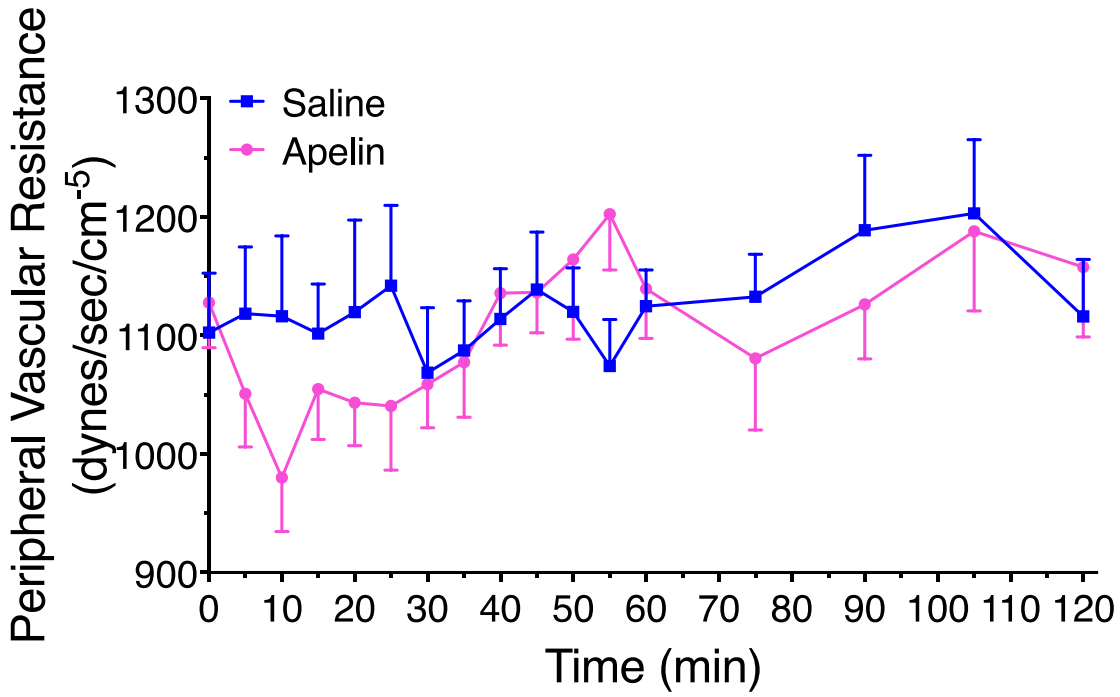


Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals in the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr^{1}]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr^{1}]apelin-13 infusion (30 nmol/min) does not cause significant change in SBP (omnibus significance P=0.201). Significantly different from saline control $P \leq 0.05$.

No significance was noted in post-hoc analyses with Bonferroni adjustments.

Figure 5.8 Effect of two-hour infusion of [Pyr[1]]apelin-13 (30 nmol/min) compared with 0.9 % saline control on peripheral vascular resistance (PVR) in T2DM patients



Measurements were taken at baseline and at 5 minute intervals during the first hour, followed by 15-minute intervals in the second hour. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr[1]]apelin-13 infusion (30 nmol/min) causes significant decline in PVR (omnibus significance P=0.01). Significantly different from saline control $P \leq 0.05$.

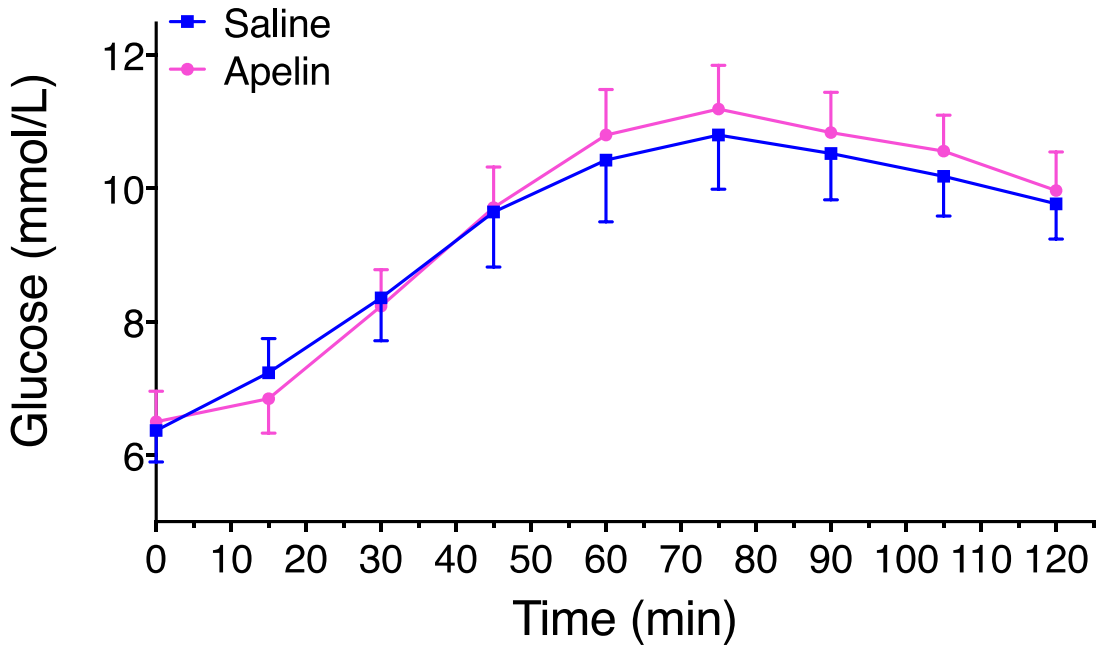
No significance is noted in Bonferroni adjustments.

5.6.3 Metabolic effects of 2-hour [Pyr^{1}]apelin-13 infusion in T2DM patients

Figures 5.9–5.11 represent metabolic changes (glucose, insulin and C peptide) in nine (N=9) T2DM patients following systemic two hour [Pyr^{1}]apelin-13 infusion (30 nmol/min) measured on dosing visits with mixed meal; V4 and V5. Log transformed values of the skewed variables (C peptide and insulin) were used for the 2-way repeated measure ANOVA analyses. Median and interquartile range [IQR] of insulin and C peptide are presented in Table 5.2 to complete data presentation.

A prolonged 2 hour [Pyr^{1}]apelin-13 infusion (30 nmol/min) does not cause significant overall change in blood glucose value (ANOVA, omnibus significance P=0.403; Figure 5.9), log transformed insulin (ANOVA omnibus significance P=0.07; Figure 5.10) nor log transformed C peptide value (ANOVA, omnibus significance P=0.076; Figure 5.11). There is no significance noted in *post-hoc* Bonferroni adjustments for any of the tested variables (glucose, log insulin, log C peptide).

Figure 5.9 Effect of two-hour infusion of [Pyr[1]]apelin-13 (30 nmol/min) compared with 0.9 % saline control on glucose in T2DM patients

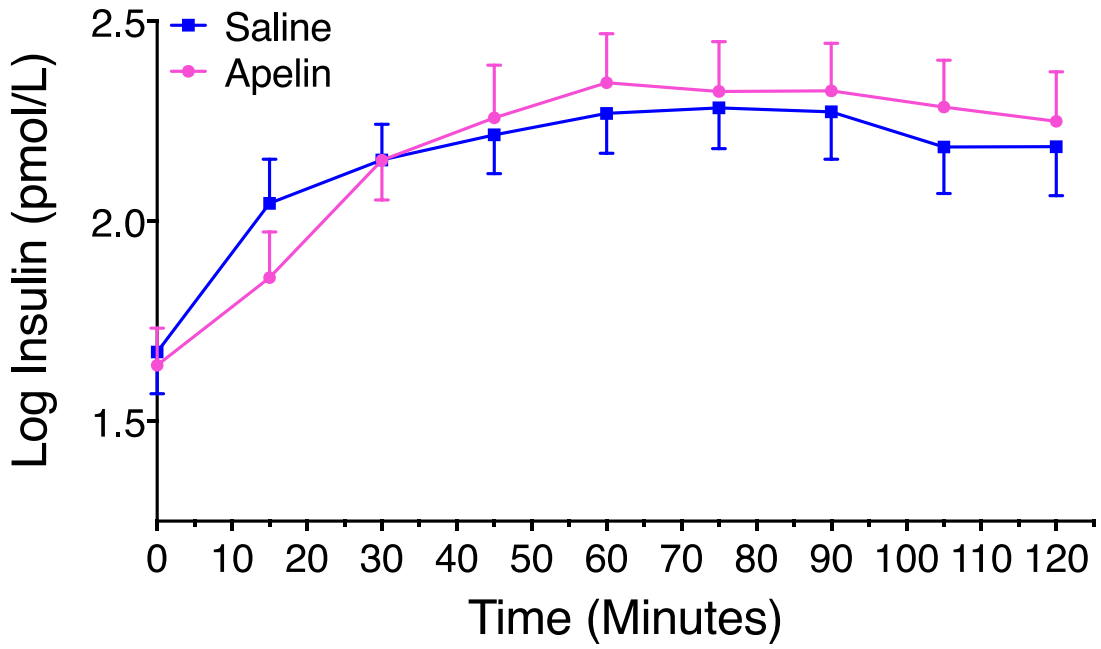


Measurements were taken at baseline followed by 15 minute intervals throughout the 2 hours. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr[1]]apelin-13 infusion (30 nmol/min) does not cause overall significant change in blood glucose (omnibus significance P=0.403). Significantly different from saline control $P \leq 0.05$.

No significance is noted in *post-hoc* Bonferroni adjustments.

Figure 5.10 Effect of two-hour infusion of [Pyr[1]]apelin-13 (30 nmol/min) compared with 0.9 % saline control on insulin in T2DM patients

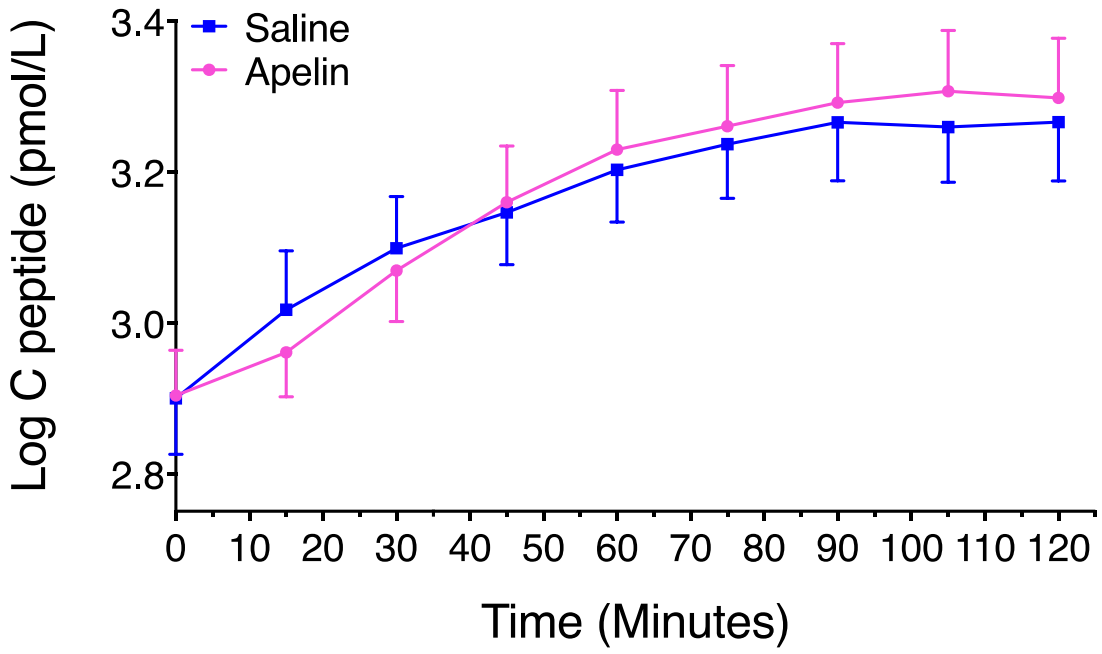


Measurements were taken at baseline followed by 15 minute intervals throughout the 2 hours. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. Log transformed insulin values were used as variables were skewed. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2-hour [Pyr[1]]apelin-13 infusion (30 nmol/min) does not cause overall significant change in log insulin value (omnibus significance P=0.07). Significantly different from saline control $P \leq 0.05$.

No significance is noted in *post-hoc* Bonferroni adjustments.

Figure 5.11 Effect of two-hour infusion of [Pyr[1]]apelin-13 (30 nmol/min) compared with 0.9 % saline control on C peptide in T2DM patients



Measurements were taken at baseline followed by 15 minute intervals throughout the 2 hours. Significance was determined by 2-way ANOVA repeated measures, with *post hoc* testing with Bonferroni's corrections. Log transformed C peptide values were used as variables were skewed. The line chart represents MEAN±SE. The pink line represents [Pyr[1]]apelin-13 and the blue line normal saline. Number of participants is N=9.

Prolonged 2 hour [Pyr[1]]apelin-13 infusion (30 nmol/min) does not cause overall significant change in log C peptide value (omnibus significance P=0.076). Significantly different from saline control $P \leq 0.05$.

No significance is noted in *post-hoc* Bonferroni adjustments.

Table 5.2 Study 5 dosing visit (V4-5) metabolic parameters at [Pyr[1]]apelin-13 dose 30 nmol/min and normal saline

Treatment	Apelin 30 nmol/L		Normal saline	
Variable (median, [IQR]) / Time/min	Insulin (pmol/L)	C peptide (pmol/L)	Insulin (pmol/L)	C peptide (pmol/L)
0	49 [25-68]	885 [855-994]	55 [35-72]	879 [849-1040]
15	86 [36-123]	1060 [826-1100]	136 [89-195]	1220 [928-1340]
30	178 [97-225]	1310 [1100-1550]	135 [129-204]	1480 [1120-1650]
45	224 [64-370]	1670 [1060-1970]	169 [138-251]	1590 [1150-1960]
60	201 [128-479]	1750 [1440-2580]	193 [149-282]	1720 [1290-2330]
75	232 [95-458]	1990 [1460-2720]	210 [125-244]	1920 [1240-2390]
90	224 [107-438]	2090 [1700-3020]	237 [119-281]	2180 [1260-2740]
105	212 [146-339]	2190 [1880-3130]	159 [138-234]	2300 [1480-2620]
120	233 [137-353]	1970 [1940-2910]	185 [138-278]	2330 [1500-2570]

At dosing visit V4-V5 each participant received a 2 hour infusion of [Pyr[1]]apelin-13 at 30 nmol/min or normal saline (control). Insulin and C peptide data are not normally distributed and are presented as median and interquartile range [IQR].

5.6.4 Safety and tolerability study results

All study participants completed four dosing visits in full and remained haemodynamically stable without pathological heart rhythm changes recorded on ECG or cardiac telemetry. Blood glucose levels have not reached the hypoglycaemia threshold (Section 2.4.3.3). Adverse events (AEs) patients experienced during the Study are shown in Table 5.3 below. There was no AEs directly related to [Pyr^{1}]apelin-13 infusion. One participant one hour into the infusion experienced diarrhoea, likely caused with previous mixed meal ingestion. All AEs fully resolved.

Table 5.3 Tolerability of [Pyr^{1}]apelin-13 infusions in participants with Type 2 diabetes mellitus

Adverse event severity	Apelin dose	Adverse event	Causality	Events N=4
Moderate	30 nmol/min	acute onset of chronic shoulder pain	not related	1
		diarrhoea	probably related	1
Mild	30 nmol/min	clammy skin	unlikely related	1
		common cold	not related	1

5.7 Discussion

The present “proof of concept” study has been designed to demonstrate for the first time the apelin cardiovascular and insulin sensitising effects in T2DM patients, which has not been studied before. The Study 3 results showing an increase in CI and reduction in PVR are in agreement with previously published cardiovascular research in HF patients (Barnes *et al.,* 2013) and the results from Study 2. However, unlike in Study 2, the results in T2DM patients do not show significant changes in insulin sensitivity.

5.7.1 Safety/Tolerability of [Pyr^{1}]apelin-13 in T2DM patients

This study showed, as expected, that systemic infusion of [Pyr^{1}]apelin-13 at dose 30 nmol/min remained safe and well tolerated in T2DM patients as was the case in Study 2 participants.

5.7.2 Cardiovascular effects of [Pyr^{1}]apelin-13 at dose 30 nmol/min in T2DM patients

The main cardiovascular finding in T2DM patients on standard of care medication was that [Pyr^{1}]apelin-13 (30 nmol/min) resulted in a significant short-term increase in SVI, CI and a reduction in PVR without causing significant changes in MAP or HR over a 2 hours infusion time.

Following pre-clinical research proving apelin to be a potent inotrope, as discussed in Chapter 4 discussion (Section 4.7.2), once again it can be suggested that a recorded increase in CI and SVI is not only secondary to vasodilatory changes but can partially be attributed to probable positive *in-vivo* inotropic actions (Maguire *et al*., 2009).

Despite the fact that we noted transiently increased HR in Study 2 participants within the first five minutes of the infusion, as would be typical for a vasodilating agent, that effect has not been noted in T2DM patients. Other than possible presumption of T2DM related cardiac autonomic dysfunction, affected by suboptimal diabetes control and approximate average 10 years disease duration in this participant group, the reason for this is unclear (Tarvainen *et al.,* 2014). Notably, none of the diabetic individuals has been on HR control treatment which would be expected to contribute to alternated chronotropic response.

Based on the clinical features of T2DM participants, as summarised in Table 5.1 (history of T2DM, smoking, uncontrolled or untreated dyslipidaemia and arterial hypertension), it can be speculated for them to have a degree of endothelial dysfunction, although no formal

endothelial function assessment has been done (Poredos *et* Jezovnik, 2013). However, a small but significant vasodilatation within the first 15 minutes caused by [Pyr^{1}]apelin-13 (30 nmol/min) has been recorded. That discovery is again in agreement with both Study 2 and previously published studies conducted in healthy volunteers and HF patients (Brame *et al.,* 2015; Japp *et al.,* 2010; Barnes *et al.,* 2013). The preservation of the vasodilatory response in T2DM patients is somewhat surprising as it is well established in experimental models and in T2DM patients that there is impaired endothelium-dependent vasodilation (mediated for example by nitric oxide-NO) in this condition (De Vriese *et al.,* 2000). These Study 3 results could therefore be explained by preclinical data showing that in conditions of endothelial dysfunction where vasoconstrictor tone to, eg, ET-1, is enhanced and NO production is attenuated, apelins have the potential to contribute to beneficial vasodilatation in an NO-independent manner (Maguire *et al.,* 2009).

Although apelin decreased MAP in Study 2 participants, there was no equivalent significant decrease in T2DM patients. The reason for this is unclear, especially as we noted anticipated increases in CO and PVR reduction in this group of participants. None of the former Study 2 participant group were on concomitant medications and only three of the T2DM patients were on anti-hypertensive therapy, suggesting drug interferences would not be a factor contributing to this difference.

5.7.3 Metabolic effects of [Pyr^{1}]apelin-13 at dose 30 nmol/min in T2DM patients

This Study compared the postprandial metabolic effects of continuous two hour [Pyr^{1}]apelin-13 infusion and placebo in T2DM patients. Unlike in Study 2 participants, we were unable to demonstrate significant overall changes in metabolic control, as apelin administration resulted in slight, but not significant change of measured metabolic parameters; insulin and C peptide in T2DM patients.

It is reasonable to presume that not only diagnosis of T2DM, but also some phenotypic differences between Study 2 and Study 3 participants could result in a different degree of insulin resistance (IR), which could explain the differences in metabolic responses alongside the same dose of apelin infusion. Although both participants groups were comparable by BMI, T2DM patients on average had less body fat percentage and higher waist circumference (Table 5.1). In addition, there could be differences within the T2DM group itself where heterogeneity in both beta cell function and IR can significantly blunt the ability to demonstrate relevant differences between individual subjects with respect to an intervention.

Secondly, we measured metabolic parameters over two hours; it is possible the effects might have occurred over a much longer time period and been accentuated with repeat dosing. Unless effects of apelin infusion would be explored over the course of days or even weeks we would not know the definitive answer. Further studies are required to determine whether there are long term effects on insulin sensitivity following repeated rather than single dosing with the peptide.

Also, in the absence of any previous metabolic studies, power calculations were based on published data for the detection of significant changes in cardiovascular parameters. However, T2DM patients differ in disease duration, concomitant comorbidities and choice in used antidiabetic agents. In addition to significant variability in metabolic properties, they also may have significant variability in baseline cardiovascular properties. All that highlights the need for greater participant numbers than in our study, which may be required to detect significant changes for metabolic findings.

Lastly, it has been speculated that apelin resistance may occur because of high endogenous apelin levels noted in obese individuals with or without diabetes (Bertrand *et al.*, 2015). Nevertheless, Gourdy *et al.,* was able to show significant improvements in insulin sensitivity in insulin resistant overweight individuals following apelin infusion. However, until clarification of existence and role of apelin resistance in diabetes, we cannot dismiss that to be another potential reason for lack of metabolic responses in patients with diabetes.

Although there was no overall significant change in metabolic parameters in T2DM, intriguingly in post-hoc testing we observed there is a starting 15-minute delay in expected increase in glucose and C peptide, which could lead to a hypothesis that there is an element of delayed gastric emptying in the T2DM patient group. Experiments show apelin-13 inhibits gastric motility and delays gastric emptying in preclinical rat and mice model (Lv *et al,* 2011; Bulbul *et al.,* 2018). As observations raising from this Study are in agreement with aforementioned preclinical work, further human studies are needed to clarify this observation (Section 5.9 and 6.2).

Altogether, T2DM patients present a challenging group to study as they are variable in relation to treatment and duration of disease, which combined with a relatively small sample do not allow us to make a final conclusion regarding metabolic responses to apelin infusion in T2DM patients at this research stage.

5.8 Study limitation

As previously acknowledged, the number of T2DM participants was chosen to match Study 2 tested subjects with increased BMI as at the time of the study design there were not any reported human metabolic studies data. Therefore, although this study was powered and statistical power was increased by cross-over design, data analyses should be interpreted with the acknowledgement that T2DM patients present a very heterogeneous patient group and increased sample may be needed.

5.9 Study conclusion

In conclusion, our results show acute systemic [Pyr^{1}]apelin-13 infusion caused an increase in CI in T2DM patients, which together with an observation of possible delayed gastric emptying represents new data in this population.

The Study cardiovascular results show acute systemic [Pyr^{1}]apelin-13 infusion caused an increase in CI and peripheral vasodilatation without affecting HR in T2DM patients. These new data identify a potential new therapeutic role of apelin in causing a beneficial hemodynamic profile in diabetic patients. Although these cardiovascular findings were obtained in T2DM patients with healthy hearts, they can be applied in the future to guide further studies in diabetics with cardiomyopathy. Based on our data and previous apelin research under heart failure conditions (Barnes *et al.,* 2013) we hypothesise apelin in T2DM patients with or without HF could be used alone or added on existing treatments without concerns of severe hypotension or reflex tachycardia. Inotropes in failing hearts are known to cause pro-arrhythmogenic effects with an increased mortality risk (Mebazaa *et al.*, 2010; Mebazaa *et al.*, 2018; Maack *et al.*, 2019; Ahmad *et al.*, 2019). We did not note any arrhythmias following acute infusion of high apelin doses although further safety evaluation is needed for long-term apelin receptor agonism.

In addition, we observed a potential delay in gastric emptying in T2DM patients. Repeat dosing, insulin clamping experiments, intravenous apelin and glucose tolerance tests alongside measurements of incretins in larger patient cohort (Section 6.2) are required to further explore the role of apelin in T2DM and to elucidate the overall clinical effect of apelin on metabolic status.

Chapter 6:

Thesis conclusions and future directions

6.1 Thesis conclusions

In addition to having a potential role of T2DM and HF biomarker, the apelin/APLNR system has a major potential therapeutic strength. Worldwide growing obesity and insulin resistance are increasing the risk of T2DM and cardiovascular diseases, both separately and in combination. Since apelin and apelin agonists act on the metabolic and the CV system, they could be considered as an interesting future pharmacological option in T2DM patients. Apelin has been thoroughly investigated in preclinical models, but to date has not yet been clinically evaluated in T2DM patients. Results of previously reported clinical research in overweight insulin resistant men describe apelin as a potential insulin sensitiser (Gourdy *et al*, 2018) and as an agent which is inducing vasodilation in healthy volunteers and HF patients (Japp *et al.,* 2010; Barnes *et al*., 2013).

This is the first proof of concept study that has been designed to examine *in vivo* cardiovascular and insulin sensitising effects of 2 hour systemic [Pyr^{1}]apelin-13 infusion in patients with T2DM to help improve our understanding of the potential use of apelin as a novel therapeutic agent in T2DM.

This thesis describes a series of experiments designed to establish the dose of [Pyr^{1}]apelin-13 that is likely to produce cardiovascular and metabolic effects in a cohort of patients with T2DM (Study 3). Studies were at first undertaken in healthy volunteers (Study 1) and in the group of increased BMI (overweight/obese) participants (Study 2) who served as a model of insulin resistance. The aim of those studies was to establish safety and tolerability, alongside determining cardiovascular and metabolic dose responses at increasing [Pyr^{1}]apelin-13 doses, prior to further testing in T2DM patients.

In the first (Study 1) pilot study, a cohort of healthy participants was studied in order to establish the safety and tolerability of [Pyr^{1}]apelin-13 infused in three escalating doses (30, 90 and 135 nmol/min respectively). Safety and tolerability were assessed by recording subjective symptoms, measurements of CV vital parameters (BP and HR), ECG monitoring, cardiac telemetry and by taking glucose measurements for early detection of hypoglycaemia. To the best of my knowledge Study 1 tested the highest literature reported doses of infused [Pyr^{1}]apelin-13, and has not shown any safety or tolerability concerns within that dose range, which presents the main finding of this study. Unfortunately, due to not frequent enough CV measurements I have not collected the data which would have been used for more detailed analyses of CV variables changes. Therefore, the frequency of CV time points has been corrected in the later Studies 2 and 3. Based on the main study finding of good tolerability and safety in Study 1, lowest (30 nmol/min) and highest (135 nmol/min) [Pyr^{1}]apelin-13 doses were selected to be further used in Study 2. These Study participants were metabolically healthy,

hence metabolic measurements were made for safety monitoring rather than for the anticipated changes in insulin sensitivity.

Study 2 was an important study to undertake as it was designed for [Pyr^{1}]apelin-13 dose selection to be further tested in Study 3 T2DM patient experiments. Study 2 was to detect metabolic and CV effects of lower (30 nmol/min) and higher (135 nmol/min) [Pyr^{1}]apelin-13 doses in two groups of overweight/class I obese but otherwise healthy participants. In addition to CV and metabolic measurements, it provided more [Pyr^{1}]apelin-13 safety and tolerability data for that dose range. Despite study limitations that formal IR measurements could not have been done due to missing laboratory data, participants were clinically presumed to be insulin resistant on the basis of their phenotype with combination of increased weight, body fat and waist circumference. Following systemic infusion, [Pyr^{1}]apelin-13 in dose 30 nmol/min increased CI, reduced PVR and MAP were recorded. HR was noted to be transiently increased, as part of the physiological reaction to vasodilation. Those CV findings were in keeping with research data reported by Barnes *et al.,* in prolonged [Pyr^{1}]apelin-13 infusions (Barnes *et al.,* 2013). After mixed meal challenge, [Pyr^{1}]apelin-13 significantly reduced plasma C peptide in these participants indicating reduction in IR. That discovery was in agreement with previously conducted work by the other group (Gourdy *et al.,* 2018) however this study conducted a different type of research experiment. Metabolic findings in this study were tested alongside MMTT to mimic physiological process in the body, whilst Gourdy *et al* have reported their data in hyperinsulinaemic-euglycaemic experiments. [Pyr^{1}]apelin-13 dose 135 nmol/min has not induced sustained CV nor metabolic effects, most likely due to APLNR desensitisation and therefore was not further used in the upcoming studies.

In the final Study 3, [Pyr^{1}]apelin-13 dose 30 nmol/min was tested in main, T2DM study cohort. This cohort consisted of insulin resistant T2DM patients, treated with per oral anti-diabetic treatment. Following systemic infusion, [Pyr^{1}]apelin-13 increased CI and reduced PVR due to the vasodilation effect, but other than the reasons of presumed cardiac autonomic dysfunction and an anticipated element of endothelial dysfunction, [Pyr^{1}]apelin-13 had no effect on HR nor MAP. After mixed meal challenge, [Pyr^{1}]apelin-13 did not quite reach significance (P=0.07) in changes of plasma C peptide and insulin levels. Although the study was sufficiently powered to pair the number of participants in Study 2, I acknowledge that T2DM patients in this group have been very clinically heterogeneous. Therefore, lack of reduction in C peptide may be attributed to the selected patient sample rather than to the lack of [Pyr^{1}]apelin-13 metabolic effects in this group. Despite the fact that C peptide reduction has not been confirmed in T2DM patients, interestingly it has been observed in the shape of the [Pyr^{1}]apelin-13 curve that the peptide may potentially cause a gastric emptying delay in T2DM patients. The role of apelin

peptide in gastric mobility has been previously confirmed in animal experiments, therefore, it would be interesting to investigate this hypothesis in T2DM patients.

In conclusion, the metabolic and hemodynamic effects of prolonged [Pyr^{1}]apelin-13 infusion were tested for the first time in T2DM patients. These experimental results show acute systemic [Pyr^{1}]apelin-13 infusion caused systemic vasodilation effects, with subsequent increase of SVI and CI in T2DM patients, which represents new data in this population. Although metabolic data from preclinical studies have highlighted that [Pyr^{1}]apelin-13 may be targeting IR in T2DM, that link based on the discoveries of this study remains unclear. Despite not proving [Pyr^{1}]apelin-13 insulin sensitising properties in T2DM patients, there could be an element of gastric emptying delay in that group which requires further investigations. Reassuringly, [Pyr^{1}]apelin-13 has not caused any concerns regarding safety and tolerability in any of the tested doses or groups of participants.

6.2 Future directions towards establishing apelin/APLNR therapeutic potential in T2DM patients

[Pyr^{1}]apelin-13 is safe to use and has shown a favourable hemodynamic profile in acute infusions, separately in HF (Japp *et al.,* 2010; Barnes *et al*., 2013) and in T2DM patients. [Pyr^{1}]apelin-13 induced vasodilation can be of clinical use, especially as preclinical and clinical experiments showed that the effect is present even in conditions of endothelial dysfunction and impaired endothelial NO response (Maguire *et al.*, 2009, Shinzari *et al.,* 2017). These promising [Pyr^{1}]apelin-13 vascular effects could be beneficial in chronic HF patients with T2DM and therefore should be investigated in the cohort of T2DM patients with concomitant HF/DCM to confirm this hypothesis.

Although not proven in this study, the hypothesis of [Pyr^{1}]apelin-13 acting as an insulin sensitizer in T2DM cannot be dismissed. In Study 3 metabolic results although not reaching significance showed overall trend in reduction of log C peptide and log insulin values. In the context of the described challenges in T2DM clinical sample selection (Section 6.1), hyperinsulinemic-euglycemic clamp experiments should be conducted to provide definitive answers of the [Pyr^{1}]apelin-13 impact on IR in T2DM patients.

In intravenous glucose tolerance test (IVGTT), body size standardised glucose bolus is injected intravenously, and glucose, insulin and C-peptide concentrations are measured (Collins *et al.,* 2021). Glucose sample is bypassing digestive system and natural incretin response stimulated by oral ingestion, and test can be used for very accurate assessment of the first phase of insulin release and insulin sensitivity. IVGTT test may be performed in order to eliminate factors related to the rate of glucose absorption. As [Pyr^{1}]apelin-13 is a

vasodilator, it cannot be excluded that vasodilation interferes with the orally absorbed glucose in MM and is possibly affecting results supporting IR reduction detected in Study 2. Therefore, the proposed future work of IVGTT alongside [Pyr^{1}]apelin-13 infusion compared to a placebo would more accurately assess the effect of [Pyr^{1}]apelin-13 on insulin sensitivity.

The effect of [Pyr^{1}]apelin-13 on inducing gastric emptying delay in rodents has been described (Lv *et al,* 2011; Bulbul *et al.,* 2018). This study observed that [Pyr^{1}]apelin-13 infusion may be delaying gastric emptying by the shape of the analyses curve. [Pyr^{1}]apelin-13 impact on gastric mobility should be tested by scintigraphy or alternatively stable isotope breath test to avoid radiation (Horowitz *et al.,* 2020). Measurement of incretin hormones in repeated experiments with MMT would provide additional information on glucose physiology under physiological conditions.

In this study, diabetes medications were excluded on the study visit day to avoid drug interferences. However, in the future it would be interesting to evaluate the [Pyr^{1}]apelin-13 effects in addition to therapeutic standard of care. Although studies described in this thesis were powered, T2DM patients present a very heterogeneous population and research investigating a greater number of participants with stricter inclusion criteria should be conducted.

Apelin can also be involved in changes in appetite and weight loss as was shown in some preclinical models. Those animal data however, are contradictory in results and the apelin effects on appetite highly depend on the apelin administration route and investigated animal species (Hu *et al.,* 2021). Due to the rapid degradation of natural apelin peptide, data describing effects of apelin on weight and appetite would be possible to collect in human subjects only alongside administration of more stable apelin analogues. Those compounds with longer half-life are under development (Castan-Laurell *et al.,* 2019). Those type of experiments may underline new therapeutic approaches in obesity treatment alongside safety and tolerability of apelin analogues under chronic use.

From a metabolic and cardiovascular point, apelin presents an intriguing therapeutic option and if proven to have antidiabetic properties combined with positive cardiovascular actions this peptide could present a novel approach in treating diabetes. Due to pharmacokinetic and pharmacodynamic properties, the development of longer acting apelin analogues without receptor desensitization should be considered if the peptide would be applied in clinical use. As apelin and APLNR are broadly distributed in the body (Section 1.3.1.1) with wide spectrum of actions, that could be taken as a disadvantage. Therefore, therapeutic modulations with organ specific distribution should be developed to avoid high systemic exposure and pleiotropic actions.

Finally, ELA as a second endogenous peptide activating APLNR should not be disregarded as a future potential therapeutic option in treatment of T2DM related complications. Properties of biased signalling (Section 1.5) and longer plasma half life underline advantages in therapeutic approach. Besides ELA acting as a biomarker in DN, it is also exerting cardio- and nephro- protective properties. Hence, it should be investigated in the context of monitoring and preventing diabetes related complications. Interestingly, ELA seems to be inducing more potent vascular responses than apelin at the same dose, suggesting ELA may be more potent vasodilating agent (Murza *et al.*, 2016). However, at this research stage, it cannot be excluded that increased responses to ELA *versus* apelin are actually due to longer plasma half-life (Section 1.5). Despite that ELA plasma half-life seems to be longer than apelin, it still remains relatively short, which makes ELA unsuitable for clinical use.

So far, the role of ELA in adipose tissue and glucose homeostasis remains unclear. However, I expect that in the future that research area will be further investigated, proposing ELA to be the new focus of the apelinergic system research. Those future discoveries could be guided with extensive preclinical knowledge related to known apelin metabolic functions.

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Appendices

Appendix 1 – Abstracts

Identification of [Pyr^{1}]apelin-12 by LC-MS/MS as the main metabolite of [Pyr^{1}]apelin-13 *in vivo* in human plasma. British Pharmacology Society (BPS) 2019

Duuamene Nyimanu, Petra Sulentic, Rhoda Kuc, Philip Ambery, Lutz Jermutus, Richard Kay, Janet Maguire and Anthony Davenport

Appendix 2 – Meetings attended

Medicine Research Day	November 2016
Annual Clinical Academic Symposium	January 2017
EMIT training event	April 2017
Research day, University of Cambridge	July 2017
EMIT/MPhil research day	July 2017
Experimental medicine training day (BPS)	October 2017
EMIT training event	November 2017
EMIT training event	March 2018
MPhil and EMIT Research day	July 2018
Pharmacology day	July 2018
Cambridge-MedImmune PhD Programme in Biomedical Research	July 2018
EMIT training event	October 2018
EMIT training event	March/2019
EMIT training event	Jan 2020
AstraZeneca & University of Cambridge	November 2020
Building bridges in medical science	March 2021
ADIT conference	March 2021
Cambridge Medical Seminars	April 2021
BHF CRE Cambridge Annual Research Symposium	March 2021

Appendix 3 – Posters

EMIT/MPhil Research day, University of Cambridge	July 2017
Research day, University of Cambridge	November 2017
EMIT/MPhil Research day, University of Cambridge	July 2018
MPhil and EMIT Research day	July 2018
MedImmune PhD Colloquium	November 2018

Appendix 4 – Courses attended

Anti-Bribery course	2016
Health and safety	2016, 2020
Fire safety	2016, 2020
GSLS courses – Core skills Training program (Research Development program)	
Skills analysis survey	2016
Time management	2016
Presentation and performance	2016
Presentation and performance workshop	2016
Introduction to scientific writing lecture	2016
Introduction to scientific writing workshop	2017
First year report workshop	2017
How to be an effective researcher	2017
Core statistics training course	2020
Writing a scientific paper	2021

Personal development program

Bioinformatics	2016
Statistics	2017
Project management	2017
Research data management	2017, 2021
Bioinformatics training course – Introduction to statistical analyses	2019

Medical library courses	2016, 2021
Database searching	
Systematic literature review	
Managing bibliography	
Critical appraisal – Randomised controlled drug trials	
Good clinical practice (GCP) training	2017, 2020
GCP protocol and associated documents	
GCP Safety reporting	
GCP Conducting the trial	
GCP Trial Master Files	
GCP Applications, Agreements, Approvals	

Clinical courses

EDGE – Research database training	2018
Advanced life support (ALS)	2018
Laboratory training competency	2018
Pump training	2018
Research bloods training	2018