Characterisation of the Apelin Receptor and its Endogenous Ligands, Apelin and Elabela/Toddler, in the Cardio-Renal System

By

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Duuamene Nyimanu

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Publications

Chapters 5 and 6 of this thesis has been published (see number 6 and 7); part of chapter 3 (see number 2) is under review for publication and has been used to secure a grant for a clinical trial of apelin in chronic kidney disease (NCT03956576).


**Reviews**


Published abstracts arising from my PhD


Presentations and Conferences Attended

British Pharmacological Society Annual Meeting (Pharmacology 2019) in Edinburgh. Poster presentation


BPS 7th Focused Meeting on Cell Signalling, Nottingham 16-17 April 2018. Poster Presentation


Internal meetings


Institute of Metabolic Sciences (IMS) Student Symposium 2018. Oral Presentation

Institute of Metabolic Sciences (IMS) Student Symposium 2017. Poster Presentation
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACE2</td>
<td>Angiotensin Converting Enzyme 2</td>
</tr>
<tr>
<td>ADAM17</td>
<td>A disintegrin and metalloproteinase domain-containing protein 17</td>
</tr>
<tr>
<td>ADMA</td>
<td>Asymmetric dimethylarginine</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APELA</td>
<td>Gene encoding elabela/toddler</td>
</tr>
<tr>
<td>APLN</td>
<td>Gene encoding apelin</td>
</tr>
<tr>
<td>APLNR</td>
<td>Gene encoding apelin receptor</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine 3’,5’-monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>DDA</td>
<td>Data dependent acquisition</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Concentration required to produce 50% of maximum response</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated glomerular filtration rate</td>
</tr>
<tr>
<td>ELA</td>
<td>Elabela/toddler</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
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<tr>
<td>FDR</td>
<td>False discovery rate</td>
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<tr>
<td>GBM</td>
<td>Glioblastoma</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GLUT2</td>
<td>Glucose transporter 2</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography (tandem) mass spectrometry</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LLOD</td>
<td>Low limit of detection</td>
</tr>
<tr>
<td>LLOQ</td>
<td>Lower limit of quantification</td>
</tr>
<tr>
<td>Lox1</td>
<td>Oxidized low-density lipoprotein receptor 1</td>
</tr>
<tr>
<td>MERS</td>
<td>Middle eastern respiratory syndrome</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PAH</td>
<td>Pulmonary arterial hypertension</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline containing 0.01% Tween 20</td>
</tr>
<tr>
<td>pD₂</td>
<td>Negative log10 EC₅₀</td>
</tr>
<tr>
<td>PGC1α</td>
<td>Peroxisome proliferator-activated receptor γ coactivator 1α</td>
</tr>
<tr>
<td>PI3k</td>
<td>Phosphoinositide 3-kinases</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLCβ</td>
<td>Phospholipase C-β</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus of hypothalamus</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real-time quantitative polymerase chain reaction</td>
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<td>Severe acute respiratory syndrome coronavirus 2</td>
</tr>
<tr>
<td>SGLT1</td>
<td>Sodium glucose cotransporter 1</td>
</tr>
<tr>
<td>SGLT2</td>
<td>Sodium glucose cotransporter 2</td>
</tr>
<tr>
<td>SON</td>
<td>Supraoptic nucleus of hypothalamus</td>
</tr>
<tr>
<td>SRM</td>
<td>Single reaction monitoring</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TAL</td>
<td>Thick ascending limb of loop of Henle</td>
</tr>
<tr>
<td>TQ-XS</td>
<td>Triple quadruple mass spectrometer</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>------</td>
<td>------------</td>
</tr>
<tr>
<td>UCP1</td>
<td>Uncoupling protein 1</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
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Abstract

The apelin system is emerging as an important therapeutic target in several diseases. It comprises a G protein-coupled receptor, the apelin receptor, which binds two peptide ligands, namely apelin and Elabela/Toddler (ELA). Although apelin has been studied in some detail, little is known about the expression and function of ELA, and the renal apelin system. The aim of this study was to characterise the expression of ELA relative to apelin and apelin receptor in mouse and human tissues; to identify specific isoforms of ELA produced in human tissues and metabolites of ELA-32 in plasma and kidney homogenate, as well as determining [Pyr₁]apelin-13 metabolites generated in vivo in humans.

ELA mRNA was expressed in mouse and human brain, lung, heart and kidney, but both apelin and ELA mRNA were absent in the spleen and liver where only the receptor mRNA was detected. Additionally, the expression of the apelin system at the protein level is poorly defined in the kidney. Within the human kidney, apelin, ELA and apelin receptor were expressed from glomerulus to the collecting duct of the renal nephron with robust localisation of the receptor to juxtaglomerular apparatus involved in systemic blood pressure regulation. Importantly, in models of kidney disease, the expression of both ligands were downregulated, but the receptor was not altered. In chronic kidney disease patients, plasma levels of both apelin and ELA were elevated.

Using a highly sensitive LC-MS/MS approach, endogenous ELA-11 was detected in human tissues for the first time. Also, novel isoforms of mature ELA-32 generated in human plasma and kidney homogenates, including ELA-11, ELA-16 and ELA-19 but not ELA-21, were found. In humans in vivo, the ACE2 metabolite [Pyr₁]apelin-13₁₋₁₂ was the most abundant metabolite of [Pyr₁]apelin-13 produced. Other potentially functional metabolites including [Pyr₁]apelin-13₁₋₁₀ and [Pyr₁]apelin-13₁₋₆ were also identified.
This thesis demonstrated that Elabela/Toddler was not a renal-specific peptide as initially thought and identified enzymatically generated isoforms of ELA and apelin produced in human plasma. A novel distribution of the apelin system in human kidney was found, supporting the hypothesis that the apelin system may have other yet unexplored functions in the kidney beside regulation of renal fluid homeostasis. Taken together, these data suggest that the apelin system may be a novel therapeutic target for the treatment of cardiorenal diseases.
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Chapter 1  Introduction

1.1 Unmet Therapeutic Need in Cardiovascular and Renal Disease

Most kidney diseases develop following a renal injury, which may progress asymptotically into chronic kidney disease (CKD), characterised by sustained loss of kidney function and kidney damage. CKD has diverse aetiologies (including diabetes and hypertension) and rapidly progresses if untreated to end-stage renal disease (ESRD), kidney failure and premature death (Mills et al., 2015). Over 20% of ESRD patients on dialysis die annually, and if diabetic, the death rate is around 70% within five years of dialysis thereby making this condition deadlier than most cancers (Breyer and Susztak, 2016). According to a study of the global burden of disease 2017, global death from CKD after controlling for age rose by 41% in 2017 compared to 1990 with approximately 2-7 million deaths annually worldwide (Bikbov et al., 2020; Hirst et al., 2020). In England alone, 15% of adults aged ≥35 years have CKD and this cost the NHS approximately £1.4 billion (Hirst et al., 2020). Essentially, CKD increases the risk for cardiovascular diseases with ~40% of haemodialysis patients showing clinical evidence of cardiovascular disease (ischaemic heart disease or heart failure) (Sarnak et al., 2003). Also, CKD patients on dialysis are 10 to 30 times more likely to die of cardiovascular disease (Sarnak et al., 2003).

One of the main treatment options for ESRD patients is renal replacement therapy which, although useful, is very expensive and therefore, inaccessible to patients in many countries (Bikbov et al., 2020). Consequently, over 2 million people die prematurely from ESRD. Currently, the majority of CKD and cardiovascular disease patients are treated with either angiotensin-converting enzyme inhibitors or angiotensin-receptor blockers. Still, these drugs only slow the loss of kidney function without significantly affecting disease progression (Breyer and Susztak, 2016). Furthermore, when used as combination therapy or in combination with renin
inhibition, various undesirable effects including hyperkalaemia, hypotension and even kidney injury, are observed. Hence, there is an unmet clinical need for novel therapies that can be used to treat cardiorenal diseases.

The apelin pathway has emerged as a critical system involved in the regulation of cardiovascular and renal function. Therefore, the studies presented in this thesis were conducted to further characterise apelin, Elabela/toddler (ELA) and apelin receptor signalling pathways in the kidney with the aim to determine if targeting this receptor might be a potential novel therapeutic strategy for the treatment of cardiorenal diseases.

1.2 The Apelinergic System

The apelin system comprises a class A G protein-coupled receptor (GPCR) called the apelin receptor and its two peptide ligands, apelin and the newly discovered zebrafish peptide ELA. The apelin receptor, initially called APJ, was found by homology cloning in 1993 (O’Dowd et al., 1993), and shown to have the highest sequence homology (54%) with the angiotensin receptor type 1 (AT₁) in the transmembrane region (Pitkin et al., 2010; Yang et al., 2015). However, the apelin receptor (gene name: APLNR) did not bind the AT₁ ligand, angiotensin II; hence it was designated an orphan receptor. The human apelin receptor comprises 380 amino acids. It is conserved in many species including mouse (91% homology) and rat (89% homology) as well as zebrafish, which expressed two isoforms of the receptor namely aplnra and aplnrb (Nornes et al., 2009; Tucker et al., 2007; Zeng et al., 2007). However, these isoforms have not been detected in mammalian systems.

1.2.1 Tissue Expression Apelin Receptor

1.2.1.1 Humans

Apelin receptor is widely expressed in all human tissues receiving a blood supply, consistent with an expression on endothelial cells, which line every blood vessel.
Apelin receptors are also localised on other cell types such as cardiomyocytes (Kleinz et al., 2005). High levels of the receptor have been detected in several other tissues including brain, spleen and placenta, with lower levels in the heart, liver, lung, kidney, pancreas, small intestine, stomach and uterus (Medhurst et al., 2003). However, apelin receptor expression has not been reported in the testes or prostate, which express apelin and ELA, respectively.

In neonatal tissues, the highest expression was observed in the lung, heart and kidney with lower levels detected in the small intestine, stomach, spleen and brain (Hosoya et al., 2000). Apelin receptor is expressed at the protein level in the right and left ventricle of the heart, media and intima of muscular arteries and large elastic arteries and veins. However, in the lungs, expression was predominantly localised to vascular beds (Katugampola et al., 2001; Kleinz et al., 2005). Additionally, the apelin receptor was also shown to be present in vascular smooth muscle and endothelial cells of human kidney and cultured human endothelial cells (Kleinz et al., 2005).

1.2.1.2 Rats

The distribution of the apelin receptor in rat tissues has also been extensively studied. O’Dowd et al. (1993) first showed by northern blotting apelin receptor expression in the hippocampus, thalamus, cortex and cerebellum. Other studies have reported a similarly wide tissue distribution in the rat when compared with humans. The mRNA was detected in the cortex (including hippocampus), subcortical regions (nucleus accumbens, striatum, hypothalamus), midbrain, cerebellum and pituitary (Medhurst et al., 2003; Lee et al., 2000; Kawamata et al., 2001; De Mota et al., 2000; Hosoya et al., 2000; O’Carroll et al., 2000). High apelin receptor expression was observed in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus, where it colocalised with vasopressin and oxytocin in magnocellular neurons (Reaux-Le Goazigo et al., 2004; De Mota et al., 2004; O’Carroll and Lolait, 2003). This may support previously identified roles in the control of fluid homeostasis (Reaux et al.,
2001; Roberts et al., 2009; Hus-Cmithrel et al., 2014) and a potential role in the regulation of male and female reproduction (Kurowska et al., 2018).

At the protein level, the expression of the apelin receptor in several regions of the brain has been reported, indicating a good correlation between transcription and translation. The apelin receptor immunoreactivity was observed in both neuronal and glial cells in the rat brain (Medhurst et al., 2003), whilst [Pyr1]apelin-13 binding sites were found in the cerebellum, the basal surface of the hypothalamic diencephalon, paraventricular nucleus (magnocellular and parvocellular neurons) and dorsal surface of the thalamus (Hazell et al., 2012; Katugampola et al., 2001).

In peripheral tissues, the highest mRNA expression was found in the lung and heart. Other tissues including the kidney, skeletal muscle, placenta, thyroid gland, ovary, uterus and adipose tissues also show expression (Hosoya et al., 2000; Medhurst et al., 2003; O’Carroll et al., 2000). A high density of the protein is found in the lungs and heart with lower levels in the kidney cortex (Katugampola et al., 2001).

1.2.1.3 Mice

Receptor expression in mice has been poorly studied. In the mouse brain, low transcript levels are found in the whole brain, cerebellum, hypothalamus, hippocampus and olfactory bulb (Regard et al., 2008; Medhurst et al., 2003). However, another study using in situ hybridisation reported a restricted central distribution in the brain, with strong expression in the paraventricular and supraventricular nucleus of the hypothalamus and anterior pituitary but lower levels in the posterior pituitary (Pope et al., 2012). Peripherally, the highest expression levels were observed in the heart, followed by moderate levels in the liver, kidney, lung, skeletal muscle and spleen, with lowest levels in the testes, thymus, bladder and ovary (Medhurst et al., 2003; Pope et al., 2012).
1.2.1.4 Species Differences in Apelin Receptor Distribution

Although the central distribution of the receptor in humans and rats are very similar, this is not the case for the peripheral distribution. Whilst the receptor was not detected in the rat spleen and liver, its expression was observed in these organs in humans as well as mice (Medhurst et al., 2003). This suggests that there could be a species difference in the distribution of the apelin receptor in humans, rats and mice. Pope et al. (2012), who characterised the distribution of the receptor in mouse using in situ hybridisation and autoradiography, reported such species differences. They observed a restricted central distribution of the receptor, with receptor transcript and protein levels abundant in the hypothalamus and anterior pituitary. Rats had a more broad distribution in the central nervous system (Pope et al., 2012). The functional significance of such species difference is currently unknown and may warrant further investigation. It could partly reflect the complexity of the apelin signalling system or that the distribution was limited to regions in the hypothalamic-pituitary-adrenal axis of the mouse brain. Additionally, although Medhurst et al. (2003) using RT-PCR detected low levels of the receptor in mouse liver and testes, Pope et al. (2012) did not detect apelin receptor in these organs using in situ hybridisation and autoradiography. This discrepancy reflects the need for more studies to clarify the distribution of the apelin receptor in mouse tissues so that physiological roles can be correctly inferred and studied.

1.2.2 Apelin Family of Peptides

About five years after the receptor was discovered, apelin-36 was identified from bovine stomach extract as the first endogenous ligand of the human apelin receptor (Tatemoto et al., 1998). It was subsequently demonstrated that the apelin peptides were initially produced as a 77-amino acid pre-proprotein containing a signal peptide, which was removed by endopeptidases to make the proapelin, apelin-55 and eventually, the mature apelin, apelin-36 (Fig. 1.1). Although the mechanism of apelin metabolism is not fully known, several enzymes have been suggested to cleave apelin
peptides including proprotein convertase subtilisin/kexin-3 (PCSK3) (Shin et al., 2013), plasma kallikrein (Fischer et al., 2019; Wang et al., 2019), ACE2 (Murza et al., 2014; Vickers et al., 2002; Wang et al., 2016; Yang et al., 2017c) and neprilysin (McKinnie et al., 2016). Consequently, several smaller but functional apelin isoforms have been produced, including apelin-12, apelin-13, [Pyr\(^1\)]apelin-13 and apelin-17 (Narayanan et al. 2015; Yang et al. 2017a).

The apelin isoforms are widely distributed in mammalian systems. For example, [Pyr\(^1\)]apelin-13 was identified as the most predominant isoform in rat plasma and hypothalamus (De Mota et al., 2004); human heart (Maguire et al., 2009) and plasma (Zhen et al., 2013). Lower levels of apelin-17 were also identified in rat hypothalamus and plasma (De Mota et al., 2004). Interestingly, apelin-55 (pro-apelin) which was initially predicted as being inactive, was identified using liquid chromatography-mass spectrometry in mature bovine milk as the predominant apelin isoform along with apelin-22 (Mesmin et al., 2011). The authors also identified many other shorter isoforms of apelin-55 (albeit at lower levels compared to apelin-55), suggesting that the proapelin was subsequently cleaved into shorter (and more active) fragments by endopeptidases in milk. Recently, apelin-55 was shown to retain biological activity at the apelin receptor (Shin et al., 2017), further extending the range of functional isoforms.
Figure 1.1. Amino acid sequences of the apelin family of peptides. The protease that cleaves apelin-55 (proapelin) to apelin-36 (mature apelin) is not yet known, but the furin, proprotein convertase subtilisin/kexin-3 (PCSK3) is known to cleave apelin-55 into apelin-17 and apelin-13. Plasma kallikrein cleaves apelin-17 into a 14-mer, apelin-14, at the N-terminus, and may also cleave the longer isoforms, apelin-36 and apelin-55. Apelin-13, the most abundant isoform in humans undergoes further posttranslational modification to form [Pyr1]apelin-13. Angiotensin-converting enzyme-2 (ACE2) cleaves all apelin isoforms at the C-terminus to remove the terminal phenylalanine residue. The * indicates the RPRL motif suggested to be critical for binding the apelin receptor and biological activity, but most of the C-terminus (C) interacts with the receptor, especially the last phenylalanine residue. Neprilysin cleaves the RPRL motif and is the only protease that cleaves and completely inactivate apelin peptides. The physicochemical properties of the amino acid residues making up apelin peptides are colour-coded.
1.2.2.1 Apelin Expression in Humans

Apelin shares a similar wide distribution with its cognate receptor in the brain and peripheral tissues. In the brain, high apelin mRNA expression was observed in all regions including the cortex, subcortex, and midbrain as well as pituitary and spinal cord (Medhurst et al., 2003; Lee et al., 2000; De Mota et al., 2000). In the periphery, the highest mRNA expression was reported in the placenta with moderate expression levels in the heart, lung, kidney and testes; whilst lower levels were detected in the liver, skeletal muscle, pancreas, spleen, small intestine and uterus (Lee et al., 2000; Medhurst et al., 2003; Habata et al., 1999; Kawamata et al., 2001). The peptide is also expressed in large conduit vessels including coronary artery and saphenous vein, renal blood vessels, vessels of the adrenal gland, and cells of the cardiac atria and ventricles (Kleinz and Davenport, 2004). Hence, the expression of the apelin receptor in organs that do not express the ligand may suggest an endocrine function of the ligand where the secreted ligand is transported in circulation to distant sites of action.

1.2.2.2 Apelin Expression in Rats

Apelin has a similar widespread distribution in the rat brain as the apelin receptor, with high levels of apelin mRNA in olfactory tubercle and several areas of the thalamic nuclei (Lee et al., 2000). At the protein level, the most detailed characterisation of apelin expression in the brain was reported by Reaux et al. (2002). They found peptide expression in almost all brain regions including telencephalon (including the septum, amygdala), diencephalon (including the thalamus, preoptic area, and hypothalamus), mesencephalon (including grey matter, dorsal raphe, and cuneiform nucleus) and medulla oblongata. Of these regions, the hypothalamus had the highest density of apelin positive cell bodies and nerve fibres. Other studies confirmed this distribution and found increased expression of the peptide, as well as co-localisation of apelin immunoreactivity with that of vasopressin (Brailoiu et al., 2002; De Mota et al., 2004; Reaux-Le Goazigo et al., 2004) and oxytocin (Brailoiu et al., 2002), in the hypothalamic PVN and SON. In addition, apelin colocalised densely with adrenocorticotrophin
(ACTH) in corticotrophic cells and less densely with growth hormones in somatotropic cells in the anterior pituitary (Reaux-Le et al., 2007). The authors also observed high apelin receptor protein and mRNA expression in ACTH positive and negative cells of the pituitary, suggesting autocrine or paracrine actions of apelin-apelin receptor signalling in the pituitary.

In peripheral tissues, high levels of apelin have been reported in the mammary gland, heart, lung and adipose tissue (Habata et al., 1999; Medhurst et al., 2003; Kawamata et al., 2001). Moderate to low levels were expressed in the kidney, adrenal gland, intestine, ovary, skeletal muscle, vas deferens, testes and uterus (Lee et al., 2000; O’Carroll et al., 2000; Kawamata et al., 2001; Medhurst et al., 2003). In addition, Habata et al. (1999) reported increased apelin expression in pregnant and lactating rats, which peaked during parturition. They also identified the peptide in bovine milk and colostrum, as well as human milk (Habata et al., 1999). In line with this, Mesmin et al. (2011) discovered ~46 endogenous apelin peptides including apelin-13, apelin-17, apelin-22 and apelin-36 in milk and colostrum. The expression of apelin and apelin receptor mRNA were highest in the gastrointestinal tract of rats at birth and decreased postnataally. Still, the density of apelin expressing cells increased progressively in the postnatal rat stomach after weaning into adulthood (Wang et al., 2009). Endogenous apelin-13 and apelin-17 peptides have also been found in rat hypothalamus (De Mota et al. 2004).

1.2.2.3 Mice

To date, only one RT-qPCR study had investigated the distribution of apelin in mouse tissues. This study found the highest mRNA expression in the brain, with moderate levels in the heart, kidney and lungs whilst lower levels were expressed in the testes, uterus, muscle, spleen and ovary (Medhurst et al., 2003).
1.2.3 Physiological Roles of Apelin at the Apelin Receptor

1.2.3.1 Apelin Receptor Signalling

In CHO-K1 cells stably expressing the mouse apelin receptor, apelin inhibited forskolin-induced cAMP accumulation, resulting in the phosphorylation of extracellular signal-regulated kinases (ERK) and protein kinase B (Akt) suggestive of signalling via inhibitory Gα protein pathway (Gα_i) (Hosoya et al., 2000; Masri et al., 2002). This was confirmed in another study which showed that apelin receptor signalling was sensitive to pertussis toxin, an inhibitor of Gα_i signalling downstream of G protein-coupled receptors (Masri et al., 2006). A pertussis-toxin insensitive but PLCβ and PKC-mediated calcium flux through Gα_q coupling were proposed in cardiomyocytes (Szokodi et al., 2002), as well as β-arrestin recruitment and desensitisation via clathrin-mediated endocytosis (El Messari et al., 2004). Signalling via both Gα_i and Gα_q pathways have been observed in adipocytes (Yue et al., 2011), while in endothelial cells (HUVECs), the apelin receptor was shown to signal by coupling Gα_13 resulting in activation and cytoplasmic translocation of histone deacetylases type 4 and type 5 (Kang et al., 2013). The signalling pathways associated with apelin receptor have been summarised in figure 1.2.

Apelin receptor is also known to signal via the β-arrestin pathway beside β-arrestin-mediated receptor desensitisation. Scimia et al. (2012) showed that β-arrestin-mediated apelin receptor signalling was involved in pathological cardiac remodelling and hypertrophy, which was independent of apelin receptor ligands but stretch mediated. Importantly, although both apelin and stretch induced ERK1/2 phosphorylation, pertussis toxin inhibition only blocked the response to apelin but not stretch. However, deletion of β-arrestin-1 and -2 abrogated apelin receptor-mediated increase in cardiac hypertrophy (Scimia et al., 2012), suggesting that apelin receptor signalling via the β-arrestin pathway may be detrimental to the heart. Additionally, a more recent study not only identified two phosphorylation sites in β-arrestin1 and three in β-arrestin2 responsible for interaction with the apelin receptor but also showed that mutation of Ser^339 residue in apelin receptor abolished interaction with GRK and β-
arrestin1/2 upon ligand stimulation and disrupted β-arrestin-mediated ERK1/2 activation (Chen et al., 2020). Moreover, point mutation resulting in the substitution of Ileu\textsuperscript{107} with alanine in apelin receptor was not only shown to abolish β-arrestin recruitment following ligand stimulation of the receptor but also prevented diet-induced metabolic dysfunction in mice (Li et al., 2020a).

**Figure 1.2. Proposed signalling pathways activated by the apelin system.** Ligand binding results in Gα\textsubscript{i}-mediated inhibition of cAMP production and vasodilation in blood vessels or Gα\textsubscript{q}-mediated increase in cardiac contractility and cardiac output. The metabolic roles have been proposed to be mediated via Gα\textsubscript{i}/Gα\textsubscript{q} pathway. DAG, diacylglycerol; PIP\textsubscript{2}, phosphatidylinositol 4,5-bisphosphate; IP\textsubscript{3}, inositol triphosphate; PI3K, phosphatidylinositol-3 kinase; eNOS, endothelial nitric oxide; NO, nitric oxide; PLC, phospholipase C; GRK, G protein-coupled receptor kinase; NHE, Na+/H+ transporters.
The cardiovascular effects of apelin are well studied and have been reviewed extensively (see Marsault et al., 2019; Mughal and O’Rourke, 2018; Pitkin et al., 2010; Read et al., 2019a). Apelin is the most potent endogenous inotropic agent yet reported with EC$_{50}$ in the picomolar range in human and rat cardiac tissues (Maguire et al., 2009; Szokodi et al., 2002). The inotropic effects of apelin are independent of angiotensin II, endothelin-1, catecholamines and nitric oxide release but may be mediated by activation of the sodium hydrogen exchanger (Japp and Newby, 2008). Stimulation of sodium hydrogen exchanger (NHE) causes increased intracellular alkalinisation following increased extracellular transport of H$^+$ and sensitisation of cardiac myofilaments to intracellular Ca$^{2+}$ (Karmazyn et al., 2001). This increases intracellular pH, but cytoplasmic Ca$^{2+}$ levels are unchanged, and studies involving patch-clamp recordings show that apelin treatment did not alter voltage-gated Ca$^{2+}$ channels in cardiomyocytes (Farkasfalvi et al., 2007; Szokodi et al., 2002).

In isolated cardiomyocytes, apelin treatment increased NHE activity whilst in rat hearts, apelin-induced positive inotropy was markedly attenuated by a specific inhibitor of NHE (Farkasfalvi et al., 2007). Therefore, this supports the hypothesis that apelin-mediated inotropic effects involve increased sensitivity of myofilaments to Ca$^{2+}$ rather than increasing intracellular Ca$^{2+}$ concentration. However, other studies in vitro and in vivo in rat hearts suggested that the inotropic response to apelin was sensitive to NHE and sodium-calcium exchanger (NCX) inhibition (Dai et al., 2006; Szokodi et al., 2002), suggesting a potential role of increased intracellular calcium in cardiomyocytes (Fig. 1.3). It remains to be determined whether the increased intracellular Ca$^{2+}$ is an indirect
cellular response to activated NHE, which increases Na\(^+\) concentration within the cell resulting in the stimulation of reverse mode NCX (Kentish, 1999).

Apelin modulates vascular tone \textit{in vivo}, decreasing blood pressure when infused into rats and dilating resistance vessels when infused into human forearm (Japp et al., 2008; El Messari et al., 2004). These responses are mediated by the production of nitric oxide (NO), although a NO-independent, prostanoid dependent vasodilation in humans has been reported (Maguire et al., 2009). \textit{In vitro} apelin causes NO-dependent vasodilation of human splanchnic artery (Salcedo et al., 2007), and vasoconstriction in human saphenous veins or endothelium-denuded vessels by direct action on vascular smooth muscle cells (Fig 1.3) whilst also acting as a potent angiogenic factor and mitogen of endothelial cells (Maguire et al., 2009; Pitkin et al., 2010). Apelin also promotes the proliferation of endothelial cells (Alastalo et al., 2011) and enhances angiogenesis via activation of VEGF signalling and sirtuin-3 expression (Zeng et al., 2014).
Figure 1.3. Signalling cascade mediating cardiovascular responses to apelin in the vascular endothelium (VEC) (A) and vascular smooth muscle cells (VSMC) (B). (A) In blood vessels with an intact endothelium, apelin signals via activation of ERK, PI3K or AMPK to stimulate endothelial nitric oxide synthase (eNOS)-mediated release of nitric oxide (NO) and prostacyclin (PGI). Prostacyclin or NO acts on VSMCs to produce vasodilation. (B) In the absence of vascular endothelium, apelin actions on apelin receptors expressed on VSMCs, results in the rise of intracellular calcium and vasoconstriction. Modified from Read, C., Nyimanu, D. et al., (2019).

1.2.3.3 Control of fluid homeostasis: Central and Renal

The expression of apelin receptor and apelin in the magnocellular neurons of hypothalamic PVN and SON expressing vasopressin and its receptors suggest a role for the apelin pathway in the control of fluid balance centrally. This expression profile
leads to a hypothesis that apelinergic and vasopressinergic signalling may inversely regulate each other in response to osmotic or volemic stimuli. Lactating animals show increased activity of the magnocellular vasopressin neurons resulting in increased synthesis and release of vasopressin to preserve water for maximal milk production (Teruyama and Armstrong, 2002), and so provided a convenient model to demonstrate the effects of apelin on vasopressin activity and control of fluid homeostasis. Intracerebroventricular injection of apelin in lactating mice inhibited the phasic electrical activity of vasopressin-expressing magnocellular neurons resulting in decreased plasma vasopressin level and increased diuresis without affecting sodium and potassium excretion (De Mota et al., 2004; Reaux et al., 2001). This was further demonstrated in dehydrated rats characterised by increased systemic vasopressin release and depletion of its hypothalamic stores. Goazigo et al., (2004) observed hypothalamic accumulation of apelin and decreased plasma apelin levels, which was reversed by the injection of a selective vasopressin receptor (V₁) antagonist in dehydrated rats. Therefore, apelin may have an autocrine role in the hypothalamic control of fluid homeostasis via a direct counter-regulatory action on vasopressin activity.

At the renal level, apelin was shown to dose-dependently relax angiotensin II pre-constricted efferent and afferent arteries and increased diuresis in lactating rats, suggesting a role in the regulation of renal haemodynamic in addition to fluid homeostasis control (Hus-Citharel et al., 2008). Like the central nervous system, the mechanism involved in the regulation of fluid homeostasis in the kidney by apelin appears to be via direct inhibition of renal vasopressinergic signalling. Hus-Citharel et al., (2014) showed that apelin inhibited vasopressin receptor signalling-induced cAMP accumulation required for activation of protein kinase A (PKA) which phosphorylates and inserts aquaporin 2 (AQP2) in the apical membrane of collecting duct principal cells. In lactating rats, apelin infusion increased diuresis by decreasing the amount of AQP2 in the apical membrane of collecting duct (Hus-Citharel et al., 2014). Therefore, the diuretic effect of apelin in the kidney involved a negative regulation of vasopressin
activity at V$_2$ receptors in the renal collecting duct (Fig. 1.4). Notably, a similar counter-regulatory activity of apelin and vasopressin has been observed in humans, where a rise in plasma osmolality (caused by hypertonic saline infusion) increased levels of vasopressin and decreased levels of apelin in the plasma (Azizi et al., 2008).

**Figure 1.4.** Schematic of the collecting duct principal cell showing the mechanisms involved in the counter-regulatory signalling between apelin and vasopressin. **Right panel:** Osmotic stimuli such as dehydration (high plasma osmolarity), decreases plasma apelin and increases plasma levels of vasopressin (AVP) which binds to V$_2$ receptors on the principal cells of the renal collecting duct. This stimulates adenylate cyclase (AC) to produce cAMP from cellular ATP resulting in the activation of protein kinase A (PKA), which causes insertion of aquaporin 2 (AQP2) containing vesicles in the apical membrane. The overall effect of this is increased water reabsorption in the basolateral membrane and decreased diuresis and urine concentration. **Left panel:**
Low plasma osmolarity has the opposite effect of reducing plasma AVP and increasing apelin plasma levels. Apelin binds its receptors on the principal cell, inhibiting AC production of cAMP and hence the insertion of AQP2. This results in increased diuresis and diluted urine.

1.2.3.4 Metabolic Roles of Apelin

In animal models, intracerebroventricular infusion of apelin was shown to improve glucose homeostasis in a NO-dependent manner (Duparc et al., 2011). However, the role of apelin on central control of food intake remains controversial. Some studies did not find any effect on food intake following intracerebroventricular administration of apelin (Drougard et al., 2016; Taheri et al., 2002) whereas others have either found an increase (Valle et al., 2008) or decrease (Lv et al., 2012; Sunter et al., 2003) in food intake. Therefore, since intravenous (iv) administration of apelin did not affect food intake (Sunter et al., 2003), it is likely that the reported effects on food intake may be due to animal handling or the release of stress mediators such as corticotrophin-releasing hormone (Lv et al., 2012; Taheri et al., 2002).

Apelin and apelin receptors are also expressed on adipocytes (Boucher et al., 2005; Wei et al., 2005), suggesting a potential role for this pathway in the regulation of adipocyte function. In adipocytes, apelin expression was increased by insulin and decreased by the glucocorticoid, dexamethasone (Wei et al., 2005). Apelin reduced blood glucose by improving glucose utilisation in the adipose tissue and skeletal muscle in both mice and humans (Attane et al., 2011; Dray et al., 2008). In vitro and in vivo, apelin dose-dependently activated AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (Li et al., 2004; Long and Zierath, 2006), which regulates skeletal muscle glucose and fatty acid metabolism, resulting in the action of endothelial nitric oxide synthase (eNOS) in soleus muscle (Dray et al., 2008). The role of eNOS and AMPK in apelin-mediated glucose uptake was confirmed by deletion of eNOS and muscle-specific loss of AMPK in mice. Interestingly, this effect of apelin was sensitive to pertussis toxin inhibition, indicative of inhibitory G protein recruitment following
activation of the apelin receptor (Yue et al., 2010). More recently, apelin was shown to increase myofiber hypertrophy through a direct action on AMPK to stimulate mitochondrial biogenesis and regenerative pathways targeting mature myofibres and satellite cells to attenuate sarcopenia (Vinel et al., 2018).

Adipose tissue and skeletal muscle are not the only tissues where apelin stimulate glucose uptake. Xu et al., (2012) showed in vitro and in vivo that apelin increased myocardial glucose uptake and membrane translocation of GLUT4 in an AMPK-dependent manner. Additionally, in the gut apelin increased glucose flux from the intestinal lumen to the bloodstream via AMPK-mediated increase in the translocation of GLUT2 to the brush border of enterocytes and decreased protein expression of SGLT1 (Dray et al., 2013). Interestingly, glucose ingestion in these mice also resulted in increased luminal secretion of apelin, suggesting that at least in the gut apelin-mediated glucose reabsorption is induced by the presence glucose of in the intestine.

A role has also been proposed for apelin in the control of lipid metabolism and energy expenditure. Apelin inhibited isoproterenol-induced lipolysis via activation of G\textsubscript{q}\textsubscript{1} and G\textsubscript{q}\textsubscript{i} signalling downstream from the apelin receptor (Yue et al., 2011). A subsequent study demonstrated the mechanism involved decreased free fatty acid release via activation of AMPK and increased production of perilipin which surrounds lipid droplets thereby preventing their metabolism by lipases (Than et al., 2012). Apelin also decreased whole-body weight gain, serum leptin and triglycerides and increased serum adiponectin without affecting food intake in mice (Galon-Tilleman et al., 2017; Higuchi et al., 2007; Nyimanu et al., 2019a). Additionally, apelin promoted the thermogenesis-mediated increase in energy expenditure by increasing body temperature, oxygen consumption, brown adipogenesis and expression of mitochondrial uncoupling protein 1 (UCP1) in brown adipocytes (Higuchi et al., 2007; Than et al., 2015). The ability of apelin to promote brown adipogenesis and energy expenditure involved activation of PI3k/Akt and AMPK pathways resulting in the expression of thermogenic transcription factors including UCP1 and peroxisome proliferator-activated receptor \( \gamma \) coactivator 1\( \alpha \) (PGC1\( \alpha \)) in brown adipocytes (Than...
et al., 2015). The concerted action of these transcription factors also increases mitochondrial biogenesis and oxidative phosphorylation.

**1.2.3.5 Other functions of apelin**

The expression of apelin isoforms in colostrum and milk, as well as expression of both ligand and receptor in the gastrointestinal tract, suggested the potential role of apelin in the regulation of gastrointestinal function. In young rats, chronic apelin administration was shown to slow gastrointestinal tract maturation (Antushevich et al., 2015). However in adult rats, apelin stimulate gastric cell proliferation, cholecystokinin (CCK), gastric acid and bicarbonate secretion (Flemström et al., 2011; Ohno et al., 2012; Wang et al., 2004). The effect on CCK secretion suggested the potential role of apelin on gastric motor functions via the inhibitory effect of CCK receptor signalling on vagal nerve activity (Bülbü et al., 2017). Indeed, exogenous apelin inhibited gastrointestinal motility (gastric emptying and colon transit) in rodents, an effect which was abolished by CCK₁/A receptor antagonist lorglumide or vagal denervation (Bülbü et al., 2017; Lv et al., 2011; Yang et al., 2010). This suggested that the effect of apelin on gastric motility was mediated by activation of CCK₁/A receptors possibly on vagal afferent nerves.

**1.2.4 Pathophysiological Roles of Apelin**

**1.2.4.1 Cardiovascular Diseases: Heart Failure and Pulmonary Hypertension**

A. **Heart Failure**

The apelinergic system has been proposed to have beneficial roles in cardiovascular diseases including heart failure, hypertension and atherosclerosis (for a detailed review see (Marsault et al., 2019; Read et al., 2019a)).

Animal studies suggest that cardiac apelin is increased in response to hypoxia via hypoxia-inducible factor-1 (Ronkainen et al., 2007), and both apelin and its receptor apelin receptor, are up-regulated in ischaemic heart failure (Sheikh et al., 2008). This
supports the emergence of the apelin receptor as a potential novel therapeutic target for the treatment of heart diseases. Apelin protects against cardiac ischaemia-reperfusion injury and inhibits cardiomyocyte apoptosis and oxidative stress (Zeng et al. 2009). The upregulation of apelin may be a protective response against the damaging effects of cardiac hypoxia caused by ischaemia-reperfusion injury. The cardioprotective effect of apelin in this context is mediated by activation of components of the reperfusion injury salvage kinase pathway (RISK) especially PI3k/Akt and MAPK signalling both in vitro and in vivo (Simpkin et al., 2007; Zeng et al., 2009). Activation of this pathway was associated with reduced infarct size, apoptosis and oxidative stress in the myocardium. Additionally, ischaemia-reperfusion injury activates endoplasmic reticulum (ER) stress, which contributes to various cardiovascular pathologies. In rats, apelin infusion protected the heart from ER stress-induced apoptosis and heart failure via PI3k/Akt and ERK1/2 phosphorylation and signalling resulting in the activation of AMPK (Tao et al., 2011).

Furthermore, several other preclinical studies have demonstrated that apelin infusion in heart failure rescued the failing heart by increasing cardiac contractility and cardiac output without affecting afterload and preload (Berry et al., 2004; Wang et al., 2013; Zhang et al., 2017). The beneficial effects of apelin in heart failure may involve angiotensin-converting enzyme 2 (ACE2). This enzyme converts the detrimental angiotensin II (Ang II) whose signalling activity cause pathologic cardiac remodelling and fibrosis, to angiotensin 1-7, a potent vasodilator which counteracts the effects of Ang II (Ferrario et al., 1997). Loss of apelin resulted in increased angiotensin II and its receptor AT1, activity in the heart and decreased ACE2 expression. However, genetic or pharmacological inhibition of AT1 signalling in apelin deficient animals increased ACE2 expression and improved cardiac function (Sato et al., 2013).

In humans, the first connection between apelin and heart failure came from Földes et al., (2003), who demonstrated that both apelin and apelin receptor levels were upregulated in heart tissues from patients with chronic heart failure caused by coronary artery disease or idiopathic dilated cardiomyopathy. In heart failure patients,
apelin levels were increased in the initial stages of the disease development and decreased in more advanced disease (Chen et al., 2003; Japp and Newby, 2008). This supports the compensatory role of apelin in restoring cardiac contractility in the early stages of heart failure, which is lost in more advanced disease. Indeed, in chronic heart failure patients, plasma apelin was positively correlated with response to cardiac resynchronisation therapy as evidenced by improved left ventricular remodelling and ejection fraction (Francia et al., 2007). Japp et al. (2010) subsequently showed that apelin decreased blood pressure and peripheral vascular resistance while increasing cardiac output in patients with heart failure. Additionally, a recent randomised controlled trial study showed that apelin had beneficial cardioprotective effects in patients with chronic heart failure by decreasing mean arterial pressure, peripheral resistance and improving cardiac function (fractional shortening and ejection fraction) (Barnes et al., 2013).

B. Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a progressive disease characterised by increased pulmonary vascular remodelling, inflammation, vasoconstriction and thrombosis or occlusion of the artery. This ultimately leads to right ventricular failure owing to the lack of drugs to cure the disease. Central to the pathology of this disease is the vascular endothelium, a vital source of apelin. Hence, it is not surprising that circulating apelin levels are decreased in PAH, although the receptor is unaffected (Kim et al., 2013; Yang et al., 2017b). Pulmonary arterial endothelial cells (PAEC) from patients also show a decreased apelin expression, increased apoptosis and hyperproliferation of pulmonary arterial smooth muscle cells (PASMC), which was rescued by exogenous apelin (Alastalo et al., 2011).

Exposure of apelin deficient mice to hypoxia exacerbated pulmonary hypertension characterised by worsened vascular remodelling and endothelial dysfunction (Chandra et al., 2011). However, treatment of models of PAH with apelin reduced right ventricular systolic pressure and hypertrophy, improved endothelial function,
decreased vascular remodelling and endothelial apoptosis (Alastalo et al., 2011; Falcão-Pires et al., 2009). Clinically, infusion of apelin in PAH patients was recently shown to have beneficial haemodynamic effects without associated hypotension and adverse effects (Brash et al., 2018), supporting the potential for targeting apelin in treating PAH.

1.2.4.2 Kidney Diseases

The circulating levels of apelin peptides in patients with CKD remain controversial. One study reported a decreased plasma apelin which was associated with cardiac function in CKD patients on dialysis (Małyszko et al., 2006). However, another study did not find any change in levels of apelin-36 in these patients although the levels of apelin-12 were significantly higher compared to healthy controls, and these did not associate with cardiovascular risk (Leal et al., 2012). In kidney allograft patients with coronary artery disease, apelin was found to be significantly decreased and independently associated with markers of endothelial dysfunction or inflammation (Malyszko et al., 2008). These studies may suggest that changes in circulating apelin in CKD were dependent on the level of endothelial damage since the endothelium is the primary source of apelin (Kleinz and Davenport, 2005). In support, a recent study investigated the levels of apelin, asymmetric dimethylarginine (ADMA) and oxidised low-density lipoprotein receptor 1 and their associations with markers of renal damage and cardiovascular dysfunction and found that both plasma apelin and ADMA were increased in haemodialysis patients compared to healthy controls (Dogan et al., 2018).

Interestingly, plasma apelin level was negatively correlated with glomerular filtration rate (used as surrogate marker of renal function). Therefore, exogenously increasing apelin levels in kidney disease patients may offer renoprotection.

Unilateral ureteral obstruction is a model of CKD caused by obstruction of the kidney resulting in decreased GFR, renal blood flow, renal atrophy and fibrosis. In this model of CKD, apelin ameliorated renal fibrosis, inflammation and myofibroblast accumulation (Nishida et al., 2012). Other animal studies showed that apelin inhibited
acute kidney injury-induced inflammation and renal fibrosis caused by increased TGFβ signalling (Chen et al., 2015a; Wang et al., 2014) and decreased inflammation in diabetic nephropathy via its actions on histone acetylation (Chen et al., 2014a). Also, administration of [Pyr1]apelin-13 in Dahl salt-sensitive hypertensive rats with end-stage heart failure inhibited cardiac dysfunction and remodelling and suppressed expression of inflammatory factors (Koguchi et al., 2012). Apelin also inhibits vascular calcification in CKD by increasing urine phosphate excretion and reducing serum phosphate levels in rats (Han et al., 2016). These studies suggest that targeting the apelin pathway may be a novel therapeutic strategy for the treatment of CKD-related pathologies.

1.2.4.3 Metabolic Diseases: Obesity and Diabetes

The critical role of apelin in the control of glucose and lipid metabolism as well as energy expenditure suggested that it could have beneficial effects in metabolic diseases like obesity and diabetes. Indeed, apelin decreased fat mass, glycemia and plasma levels of triglycerides in diet-induced obese and insulin-resistant mice (Attané et al., 2012; Dray et al., 2008). Additionally, chronic apelin infusion increased fatty acid β-oxidation in the soleus muscle of high-fat diet-fed mice (Attané et al., 2012). These may suggest that the beneficial effect of apelin in diet-induced obese mice may involve increased fatty acid metabolism and insulin sensitivity in skeletal muscle and adipocytes. Yamamoto et al., (2011), generated transgenic mice expressing apelin and showed that these mice were resistant to diet-induced obesity when fed a high-fat diet potentially via increased energy expenditure.

Interestingly, the resistance of these mice to diet-induced obesity was correlated with increased expression of angioptietin-1 and its receptor Tie 2, resulting in vascular formation in skeletal muscle. Sawane et al. (2013) have demonstrated the importance of vascular integrity in preventing obesity. They showed that diet-induced obesity in apelin deficient mice was associated with abnormal blood vessel and lymphatic enlargement and hyperpermeability, which was stabilised by exogenous apelin. In
support of these, apelin null mice had increased serum free fatty acid, glycerol, adiposity and leptin, which was ameliorated by exogenous infusion of apelin for two weeks (Yue et al., 2011).

The beneficial effects of apelin on vascular function in obesity was also confirmed in humans. In these subjects, apelin infusion improved insulin-stimulated vascular function by inhibiting angiotensin II and endothelin-1 induced vasoconstriction independent of NO (Schinzari et al., 2017). Additionally, the plasma levels of apelin in obese and diabetic patients have been extensively studied and showed to increase (Castan-Laurell et al., 2011; Chaves-Almagro et al., 2015). In a randomised control trial study of anti-diabetic drugs metformin and rosiglitazone, plasma apelin and ghrelin were increased in patients receiving the combined treatment. This increase was associated with improved glycaemic control and decreased LDL, suggesting that the beneficial effects of these drugs may involve apelin receptor signalling (Kadoglou et al., 2010). Hence, it is intriguing that the beneficial effects of apelin in the metabolic disease were translatable from animal models to humans, further reinforcing the importance of the apelin pathway as a novel therapeutic strategy.

1.2.4.4 Other Diseases

The apelin pathway has also been implicated in several other diseases, including infection by human and simian immunodeficiency viruses (SIV and HIV) and cancer. HIV-1 and SIV-1 viruses usually use CD4 and a coreceptor (usually one of the chemokine receptors CCR5 and CXCR4) for host cell entry and infection (Albright et al., 1999). The apelin receptor, which has a similar structure to CXCR4, was shown to act as co-receptor for host entry by these viruses (Edinger et al., 1998; Puffer et al., 2000), but this was blocked by exogenous apelin (Cayabyab et al., 2000; Zou et al., 2000). However, the clinical relevance of this has not been fully explored.

In various forms of cancer, the expression of apelin and apelin receptor are increased, and this has been associated with increased angiogenesis, proliferation, metastasis,
induction of cancer stem cells and drug resistance (for a detailed review see (Masoumi et al., 2020)). Apelin was shown to be a potential biomarker for normalisation of colon adenocarcinoma following treatment with the anti-angiogenic therapy, bevacizumab in mice (Zhang et al., 2016). High levels of circulating apelin were also associated with poor prognosis in renal, breast and lung carcinoma patients treated with the receptor tyrosine kinase (RTK) inhibitor, sunitinib (Uribealogo et al., 2019). Genetic deletion of apelin decreased tumour angiogenesis, inhibited tumour growth and prevented resistance to RTK inhibitors in mammary and lung cancer (Uribealogo et al., 2019).

In apelin deficient mice, orthotopically implanted with glioblastoma (GBM) cells, tumour vascularisation and growth was severely impaired compared to control cells on the apelin-wildtype background (Frisch et al., 2020). This phenotype was rescued by exogenous apelin. Loss of apelin in either the GBM cells or microenvironment significantly decreased tumour angiogenesis and vascular density in subcutaneous xenograft mice (Frisch et al., 2020). This supports a crucial pro-tumourigenic role of apelin-apelin receptor signalling in GBM through increased tumour angiogenesis and vascularisation, suggesting that apelin receptor may be a potential target to inhibit pathological angiogenesis in cancer. Indeed, inhibition of apelin receptor signalling with a novel peptide antagonist, MM54, was shown to inhibit tumour angiogenesis, growth and metastasis (Harford-Wright et al., 2017). This was corroborated in a recent study, which showed that targeting the apelin receptor signalling in GBM in combination with bevacizumab synergistically enhanced survival with an additional benefit of blocking the pro-invasive side effect bevacizumab monotherapy (Mastrella et al., 2019).

1.2.5 Genetic Knockout of Apelin and Apelin Receptor

The role of apelin and apelin receptor in the development of the cardiovascular system has been demonstrated through apelin and apelin receptor knockout mice. Kuba et al. (2007) generated apelin knockout mice and showed that although both male and female littermates were viable, fertile and appeared healthy, they developed
progressive impairment of cardiac contractility associated with systolic dysfunction with age. Conversely, apelin receptor null mice were not born in the expected Mendelian ratio and manifested severe cardiovascular developmental defects including impaired maturation of the yolk sac and embryo vasculature, aberrantly formed right ventricle and defective formation of atrioventricular cushion needed for proper development of the heart septum (Charo et al., 2009; Kang et al., 2013). Similarly, both apelin and apelin receptor mutant mice had decreased exercise capacity due to reduced sarcomeric shortening and impaired velocity of contraction in isolated cardiomyocytes (Charo et al., 2009). Interestingly, although apelin knockout mice developed progressive heart failure without any significant effect on hypertrophy in response to pressure overload (Kuba et al., 2007), apelin receptor mutant mice showed reduced cardiac hypertrophy and developed heart failure in response to pressure overload (Scimia et al., 2012). Overall, information from apelin and apelin receptor null mice studies indicate that the apelinergic system is vital to normal cardiovascular development and function, but there are unexpected discrepancies in the ligand and receptor knockout.

Similar discrepancies were observed during zebrafish development. Whilst the apelin receptor was expressed very early in the blastoderm stage and continued through gastrulation; apelin was expressed at mid gastrulation in the midline. Functionally, loss of apelin in zebrafish did not phenocopy the loss of apelin receptor during zebrafish development. Deletion of the apelin receptor, agtlr1b, resulted in a reduced number of myocardial progenitor cells and the absence of an organised heart (Scott et al. 2007; Zeng et al. 2007). However, deletion or overexpression of apelin impaired the migration of anterior lateral plate mesoderm cells during gastrulation resulting in myocardial progenitor cells not being in the correct position to receive inductive signals required for differentiation, and thus defective heart development (Zeng et al., 2007). These discrepancies in the spatiotemporal expression and function of apelin and apelin receptor may be indicative of another yet unidentified ligand for the apelin receptor, which compensated for the loss of apelin during heart development.
1.2.6 Discovery of Elabela/Toddler as a Ligand of the Apelin Receptor

Using zebrafish as a model, two research groups independently discovered a novel ligand for the apelin receptor in a region of the genome previously thought not to encode a functional gene and named it Elabela (Chng et al., 2013) or Toddler (Pauli et al., 2014). Since the name given by researchers who first discovered the novel peptide was Elabela, the International Union of Pharmacology (IUPHAR) and Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC) assigned the name Elabela or ELA to the peptide and Apela (for apelin receptor early endogenous ligand) to the gene encoding this peptide (Read et al., 2019a). Therefore, this IUPHAR and HGNC nomenclature will be used in this thesis to refer to the protein and gene.

ELA is highly expressed during gastrulation and is important for proper heart development in zebrafish (Chng et al., 2013; Pauli et al., 2014). ELA deletion resulted in severe cardiac morphogenesis defects and lack of rudimentary heart or no heart at all in embryos (Chng et al., 2013; Pauli et al., 2014). A very similar phenotype was also observed when the apelin receptor was deleted from zebrafish embryos, suggesting that both ELA and apelin receptor were linked (Chng et al., 2013; Pauli et al., 2014). Additionally, ELA was not only present at the same time and in the same cell or adjacent cells to those expressing the apelin receptor, but pharmacological experiments showed that exogenous ELA bound the apelin receptor and rescued the phenotypes of ELA-deficient embryos (Pauli et al., 2014). However, apelin is not expressed until mid-gastrulation in zebrafish, and apelin deficient embryos did not appear to show any defective phenotype (Chng et al., 2013). This suggested that ELA was a second endogenous apelin receptor ligand pivotal to the proper development of the cardiovascular system.
1.3 ELA Processing and Tissue Distribution

*Apela* gene, which is located on chromosome 4 has three exons. It is translated into a 54 amino acid peptide, which is further processed by enzymatic removal of the signal peptide to ELA-32, the mature isoform (Fig. 1.5). ELA-32 has two potential furin cleavage sites marked by dibasic residues and was therefore predicted to undergo further processing by furin into ELA-21, ELA-11 (Chng et al., 2013; Pauli et al., 2014), and recently ELA-22 (Murza et al., 2016). However, only ELA-11 has been observed endogenously in embryos expressing *apela* mRNA by mass spectrometry (Pauli et al., 2014). The C-terminal sequence of ELA peptides, especially ELA-11, is well conserved in all vertebrates, suggesting that this region was critical to biological function.

![Diagram showing amino acid sequences of ELA-32, ELA-21, ELA-11, and [Pyr1] Apelin-13]

*Figure 1.5. The amino acid sequence of ELA-32, ELA-21 and ELA-11 compared to [Pyr1]apelin-13. Both apelin and ELA have similar physicochemical properties as indicated by the colour-coding and a conserved C-terminus (C). The * indicate amino acid residues in apelin and ELA that are critical for binding to the apelin receptor. Notice that the amino acid residues required for binding are in the C-terminus of ELA*
peptides but present at the N-terminus in [Pyr$^1$]apelin-13. The longer ELA isoforms, ELA-32 and ELA-21, were predicted to have a disulphide bridge between the Cysteine residues.

Since ELA is a recent discovery, the distribution of Apela mRNA and the peptide in human and rodent tissues has not yet been thoroughly investigated. However, ELA mRNA is developmentally regulated, with the inner cell mass of the blastocyst showing highest expression, and is downregulated upon differentiation (Ho et al., 2015). Initial studies found that, in addition to its expression during embryonic development, ELA is also present in adult human kidney and prostate tissues (Chng et al., 2013; Wang et al., 2015b). Apela transcripts have been reported in human blood vessels, with the highest levels detectable in arteries compared to veins, and lower levels in the human heart and lung tissue (Yang P. et al., 2017b). At the protein level, ELA is localised to the endothelium of human heart and lungs vessels (Yang et al., 2017b).

In the rat, Deng et al. (2015) suggested that ELA was exclusively expressed in the adult kidney compared to very low levels of apelin and apelin receptor. Recently, Perjés et al. (2016) demonstrated that although ELA mRNA was detectable in the adult rat heart (albeit at lower levels compared to apelin), this expression was mainly localised to non-cardiomyocytes, especially endothelial cells and fibroblasts.

1.3.1 Developmental Role of ELA

ELA is highly secreted by human and mouse embryonic stem cells where it plays critical roles in cardiovascular development, self-renewal and apoptosis (Ho et al., 2015; Li et al., 2015). In mouse embryonic stem cells, ELA has a non-coding function where it promotes p53-mediated DNA damage-induced apoptosis by binding to and inhibiting the p53 inhibitor, heterogeneous nuclear ribonucleoprotein L (hnRNPL) (Li et al., 2015). In human embryonic development, ELA provides signals required to direct human embryonic stem cells (hESCs) toward the mesendodermal lineage by activating PI3K/Akt signalling and priming hESCs to endoderm differentiation (Ho et
This results in enhanced cell-cycle progression and optimal protein translation, thereby preventing stress-induced apoptosis in hESCs. Surprisingly, this function of ELA was proposed to be independent of the apelin receptor since the receptor is not expressed in undifferentiated hESCs (Ho et al., 2015). Hence, whilst another receptor may potentiate ELA signals in hESCs, it is not clear why ELA will act via a different receptor in these cells, and there has been no evidence to corroborate this in adults or other species.

Studies in zebrafish showed that ELA was also required for vasculogenesis, where it bound the apelin receptor to provide signals necessary for spacio-temporal migration of angioblasts to the midline during dorsal aorta and cardinal vein formation (Helker et al., 2015). Importantly, apelin receptor mutants were also unable to form these large vessels, suggesting that ELA acts via the apelin receptor to regulate vasculogenesis. They reported that when ELA wildtype angioblasts were injected into mutants, vessel formation was restored supporting a role for secreted ELA. In support, ELA was shown to control the development of coronary vasculature in mouse hearts (Sharma et al., 2017).

Coronary vasculature is formed from two progenitors, endocardial progenitors (regulated by microenvironmental signals) and sinus venosis (genetically regulated), which merge to create a functional coronary circulation. These progenitors colonise the mammalian heart; with the endocardial progenitors lining the heart lumen occupying heart muscles from inside out whilst sinus venosis feed ventricular muscle from the epicardium into the heart (Chen et al., 2014b; Red-Horse et al., 2010; Tian et al., 2014). Sharma et al., (2017) observed in ELA or apelin receptor-deficient mouse hearts that migration of blood vessels to regions populated by sinus venosis was impaired while those populated by endocardial progenitors were unaffected. Importantly, in the absence of sinus venosis due to genetic loss of ELA or apelin receptor, the heart becomes hypoxic and release factors, which stimulate endocardial progenitors to sprout and compensate for the lack of sinus venosis-derived coronary vessels (Sharma et al., 2017). Consequently, these offspring survive to adulthood with
normal cardiac function. This compensatory mechanism may in part explain some of the incomplete penetrances of embryonic lethality observed in ELA and apelin receptor-deficient mice models.

**1.3.1.1 Mouse Knockout Studies**

Since ELA was discovered in zebrafish, many studies focused on this model organism despite being evolutionary distant to humans. Two research groups generated ELA knockout mice to develop some insight into the role of ELA in mammals. These studies showed that like apelin receptor knockout mice (Kang et al., 2013), ELA knockout mice have incomplete penetrance of embryonic lethality with a subset (~10-50%) of pups surviving to adulthood (Freyer et al., 2017; Ho et al., 2017). Interestingly, Ho et al., (2017) found that over 68% of embryos died *in utero* when both the mother and foetus were deficient for ELA, suggesting that circulating ELA from maternal blood may be used by the embryos to compensate for the loss of ELA. In support, ELA was also present in the blood of pregnant mice but absent in the blood of non-pregnant mice (Ho et al., 2017). Phenotypic analysis of embryos from ELA deficient mice revealed poorly looped heart tube, reduced smooth muscle actin and pericardial oedema (Freyer et al., 2017; Ho et al., 2017). This was consistent with a role in mammalian heart development. Additionally, although the reason for the discrepancy in the percentage of surviving foetuses recorded in the two studies is unclear, the non-coding role of ELA (Li et al., 2015) and/or ligand-independent effects of apelin receptor (Scimia et al., 2012) may be responsible.

Furthermore, ELA deficient embryos presented various defects resulting from improper development of foetal-maternal circulation such as underdeveloped yolk sac vasculature, thin placental labyrinth and poorly vascularised placentas (Freyer et al., 2017; Ho et al., 2017). *Ela* null pregnant mice showed decreased vascularisation of placental labyrinth due to increased cell death and reduced differentiation rate of syncytiotrophoblasts (Ho et al., 2017). This may support a potential role of ELA in the regulation of placentation and placental development at least in mice. In support, ELA
was expressed in syncytiotrophoblasts juxtaposed to apelin receptor-expressing foetal endothelial cells, implicating a paracrine role of ELA in placental development (Ho et al., 2017). In humans, ELA and apelin receptor were expressed in trophoblasts in the first-trimester placenta and promoted differentiation of placental explants towards an invasive phenotype \textit{ex vivo} (Georgiadou et al., 2019). Hence, these studies confirm a role for ELA in the regulation of placentation or placental development in mice and humans.

ELA was thought to be the earliest expressed ligand and that its expression coincided with apelin receptor expression based on findings in zebrafish (Chng et al., 2013; Pauli et al., 2014). However, this does not appear to be true in mice. Freyer et al., (2017) investigated the spatiotemporal expression of apelin, ELA and apelin receptor in wildtype mice embryos and found that apelin was indeed the first apelin receptor ligand to be expressed in mice. However, apelin was unable to compensate for the loss of ELA, as demonstrated in apelin: ELA double knockout mice (Freyer et al., 2017). This may suggest that although apelin and ELA signal via the apelin receptor, ELA-apelin receptor signalling may involve distinct downstream effectors, which are not recruited by apelin signalling. Moreover, it may also indicate the possible involvement of alternative signalling pathways and receptors for ELA independent of apelin receptor system (Ho et al., 2015).

1.3.2 Functions of ELA in Adult Mammalian Physiological Systems

1.3.2.1 Cardiovascular Roles

ELA competes with apelin for binding in cardiomyocytes, suggesting that it was active at the adult apelin receptor. Like apelin, ELA increased cardiac contractility and induced coronary artery vasodilation in adult rat (Perjés et al. 2016; Yang et al. 2017b). Interestingly, the vasodilator role of ELA was not blocked by the NOS inhibitor, L-NAME (Wang et al., 2015), suggesting that NO was not required for ELA-induced vascular relaxation. Moreover, ELA also stimulates angiogenesis in HUVECs through
activation of the apelin receptor and induced relaxation of mouse aortic vessels ex vivo (Wang et al., 2015). Additionally, in isolated hearts and anaesthetised rats, ELA caused strong positive inotropic effects and decreased mean arterial pressure (Coquerel et al., 2017; Murza et al., 2016), which was blocked by an apelin receptor antagonist, MM54 (Coquerel et al., 2017). The decrease of mean arterial pressure induced by ELA was higher than that of apelin at the same dose, suggesting that ELA may elicit more potent vasorelaxation than apelin (Murza et al., 2016). However, it is not clear whether the observed increase in response to ELA compared to apelin was due to better plasma stability of the former, since apelin is known to have poor plasma stability (Japp et al., 2008). Additionally, ELA was shown to dose-dependently increased right and left ventricular ejection fraction, which is a surrogate measure of cardiac contractility in rats (Yang et al., 2017b).

The signalling pathways associated with ELA remain poorly understood. Using CHO cells artificially overexpressing the human apelin receptor, it was shown that ELA signalling inhibited cAMP accumulation resulting in ERK1/2 phosphorylation and induced calcium mobilisation (Wang et al., 2015), suggesting that ELA signals via Gαi and Gαq pathways at the apelin receptor. ELA also promotes rapid internalisation of the apelin receptor (Murza et al., 2016). Interestingly, Perjés et al., (2016) also found in isolated rat hearts that the inotropic effects of ELA downstream the apelin receptor was mediated by activation of ERK1/2 signalling. This was confirmed using a selective inhibitor of an upstream regulator of ERK1/2, MAPK kinase 1 and 2 (MEK1/2), which abrogated the inotropic effects of ELA but the protein kinase C inhibitor, Bis did not affect responses to ELA (Perjés et al., 2016). Scimia et al., (2012) also showed in HEK cells stably expressing the human apelin receptor that ERK1/2 activation by apelin was blocked by pertussis toxin inhibition, further suggesting that ERK1/2 phosphorylation was Gαi mediated.
1.3.1.1 Fluid Homeostasis and Kidney

ELA signalling is essential in the central and renal regulation of fluid homeostasis. Peripheral administration of ELA in rats increased diuresis and water intake without affecting food intake, which was more potent than apelin (Deng et al., 2015; Murza et al., 2016). Addition of an analogue of ELA called Apela-PA, which acted as an antagonist, inhibiting the ELA signalling activity at the apelin receptor, abolished the diuretic effect of ELA (Deng et al., 2015). Intracerebroventricular administration of ELA reduced food intake in mice by activating arginine vasopressin and corticotrophin-releasing hormone (CRH) neurons in the paraventricular nucleus (Santoso et al., 2015). However, the reason for the discrepancy between central and peripheral ELA administration on food intake is unknown, but considering that central administration induced the release of ACTH, this anorexigenic effect may have been caused by stress.

1.3.3 Pathophysiological Roles of ELA

1.3.3.1 Preeclampsia

Preeclampsia is a pregnancy disorder of unknown aetiology characterised by high blood pressure and the presence of proteins in urine. It affects around 5-8% of all pregnancies and can often lead to premature or preterm birth, maternal morbidity and mortality (Liu et al., 2011). Consistent with a role in placental development, ELA was proposed to be involved in the pathogenesis of preeclampsia. Intriguingly, ELA null pregnant mice developed features of preeclampsia, including proteinuria, hypertension and glomerular injury due to impaired placental angiogenesis (Ho et al., 2017). Exogenous administration of ELA in Ela-deficient preeclamptic mothers, rescued the phenotype without affecting embryonic development of the foetus (Ho et al., 2017). Surprisingly, apelin failed to rescue preeclamptic phenotype in these animals, suggesting that apelin and ELA may recruit different downstream effectors at least during placental development and in preeclampsia. Collectively, these observations are indicative of a potentially important role for ELA in preeclampsia and...
raise the possibility that exogenous ELA may be useful as a potential therapy in this condition.

Despite the potentially compelling evidence for the role of ELA in preeclampsia, care may be required in consideration of the clinical utility of these data because several peptides have also been implicated in preeclampsia to date. These include apelin (Wang et al., 2017), adrenomedullin (Di Iorio et al., 1998; Kanenishi et al., 2001), calcitonin gene-related peptide (CGRP) (Dong et al., 2005), neuropeptide Y (Paiva et al., 2016) and atrial natriuretic peptide (ANP) (Zhou and Wu, 2013) amongst others, none of which have made it into the clinic. Therefore, it is not clear where ELA sits in relation to all these other peptides or how this purported role of ELA in preeclampsia translates to humans considering the substantial differences between mouse and human placentas (Wirka and Quertermous, 2017). Further studies are therefore required to clarify the role of ELA in pregnancy-related complications and demonstrate its translatability to humans.

Several studies were performed to delineate the role of ELA in preeclampsia, especially in humans by measuring the circulating levels of ELA in pregnant women but produced contrasting findings. Whilst some studies did not find any correlation or changes in ELA levels in pregnant women with preeclampsia (Pritchard et al., 2018; Villie et al., 2019), others found a decrease (Deniz et al., 2019; Zhou et al., 2019) or an increase (Panaitescu et al., 2018) in circulating ELA levels in these women. Interestingly, it seems that circulating ELA levels in women with preeclampsia correlate with their BMI. For instance, Zhou et al., 2019 studied women with BMI <25, Panaitescu et al., 2018 studied women with a BMI ≥28 and Pritchard et al., studied women with BMI between 23 and 31, whilst other studies did not specify the BMI range of women involved in the study. Pregnant women with healthy BMI (<25) who will later develop preeclampsia, had decreased ELA levels in the first trimester (Georgiadou et al., 2019), but it is unclear whether at the time they developed preeclampsia their BMI had increased significantly.
1.3.1.2 Cardiovascular Diseases: Heart Failure and Hypertension

A. Heart Failure

Like apelin, ELA also has beneficial effects in heart failure and have been shown to protect against angiotensin-induced hypertension, cardiac remodelling and damage. Chronic infusion of ELA in a pressure-overload induced mice model of heart failure showed attenuated myocardial hypertrophy, fibrosis and contractility damage (Sato et al., 2017). ELA treatment was also found to decrease expression of heart failure and fibrosis genes, including TGFβ, atrial natriuretic peptide and angiotensin-converting enzyme (ACE) (Sato et al., 2017). Mechanistically, the beneficial effects of ELA in heart failure appear to be by downregulation of the transcription factor FOXM1, which upregulates ACE in stressed hearts. Surprisingly, apelin did not affect the expression of FOXM1 or ACE although it positively regulated ACE2 expression in failing hearts (Sato et al., 2013, 2017), suggesting that the beneficial effects of ELA and apelin in heart failure may involve different mechanisms despite using the same receptor.

Myocardial fibrosis plays a vital role in the pathogenesis of heart failure. In animal models, ELA was shown to lower myocardial inflammation, myocardial injury and improve cardiac function (Coquerel 2017). In a rat model of ischaemia reperfusion-induced myocardial infarction, administration of ELA was shown to restore cardiac function by reducing infarct size, improving ejection fraction and fractional shortening (Rakhshan et al., 2019). ELA also inhibited oxidative stress by increasing the activity of antioxidants, glutathione and superoxide dismutase.

The relatively short half-life of ELA (< 2mins) previously reported in rodent plasma (Murza et al., 2016), compromised its utility as a potential therapeutic agent in heart failure. Immunoglobulin Fc-fusion proteins are a well-established strategy used to improve the half-life and efficacy of drugs and have been used to develop over six FDA-approved drugs including abatacept, used to treat rheumatoid arthritis and dulaglutide (an Fc-GLP-1) for treatment of diabetes (Beck and Reichert, 2011). Xi et
al., (2019) developed an ELA-Fc fusion protein where ELA was coupled to an IgG-Fc domain (Fc-ELA-21), which extended the half-life to 44 hr without affecting biological activity at the apelin receptor. Daily administration of Fc-ELA-21 in a rat model of myocardial infarction improved cardiac function by attenuating myocardial fibrosis and apoptosis whilst promoting angiogenesis and cardiomyocyte proliferation (Xi et al., 2019).

B. Hypertension

Like apelin, the ELA-apelin receptor pathway has beneficial roles in hypertension. Interestingly, ELA levels were significantly decreased in hypertensive patients when compared to healthy controls and negatively associated with the systolic and diastolic blood pressure but positively correlated with endothelial functions (Li et al., 2020b). This was consistent with the idea that the endothelium is the primary source of circulating ELA and that exogenous ELA may be a potential therapy in hypertension. Similarly, ELA levels were decreased in cardiopulmonary tissues of PAH patients and rat models (Yang et al., 2017b). Infusion of ELA in animal models of PAH attenuated disease progression by decreasing right ventricular systolic pressure and hypertrophy, pulmonary vessel muscularization and arteriolar wall thickness without affecting systemic blood pressure (Yang et al., 2017b).

Furthermore, a recent study showed that peripheral administration of ELA-21 had anti-hypertensive effects of decreasing mean arterial pressure (MAP) and heart rate in spontaneously hypertensive and Wistar-Kyoto rats (Geng et al., 2020). However, the authors saw increased expression of ELA in the rat PVN and administration of the exogenous peptide in PVN, stimulated increased sympathetic nerve activity resulting in the increased heart rate, blood pressure and vasopressin release (Geng et al., 2020). Notably, both of these effects were blocked by apelin receptor antagonist, suggesting they were apelin receptor-dependent.
1.3.1.3 Renal Diseases

Some initial reports have implicated ELA-apelin receptor signalling in renal disease based on animal studies. Coquerel et al. (2017) showed that ELA infusion improved cardio-renal outcomes in experimental septic shock rats by improving fluid homeostasis, cardiovascular haemodynamic and ameliorated kidney dysfunction in a vasopressin-dependent manner. The authors also reported that although both apelin and ELA independently had beneficial actions, ELA posed more substantial effects than apelin. Exogenous ELA is protective in acute kidney injury where it inhibited DNA damage response, apoptosis, inflammation and injury-induced renal fibrosis (Chen et al., 2017).

In rats fed a high-salt diet, ELA treatment preserved glomerular architecture and alleviated renal fibrosis (Schreiber et al., 2016). The beneficial effects of ELA on salt-induced hypertension and kidney injury appears to involve direct action on the renin-angiotensin system. Xu et al., (2020) showed that high salt intake in Dahl Salt-sensitive rats activate prorenin receptor and intrarenal renin-angiotensin system whilst inhibiting ELA-apelin receptor signalling. This resulted in salt-induced hypertension, inflammation and kidney injury. Additionally, the prorenin receptor and soluble prorenin receptor signalling inhibited ELA and apelin expression further exacerbating hypertension. However, ELA infusion showed beneficial effects by decreasing prorenin receptor and soluble prorenin receptor expression, thereby inhibiting intrarenal RAS activation and consequently reducing inflammatory markers, renal injury and lowering blood pressure (Xu et al., 2020).

Furthermore, in a chronic streptozotocin-induced diabetic nephropathy mouse model, ELA reduced renal fibrosis and inflammation, resulting in improved kidney function (Zhang et al., 2019). It does this by activating the apelin receptor signalling resulting in the recruitment of PI3K/Akt and mTOR signalling pathways in podocytes to promote survival. Apelin also has similar beneficial effects in diabetic kidney disease by inhibiting podocyte apoptosis via ERK/Akt/p70S6K signalling (Müller et al., 2018),
suggesting that the beneficial effects of ELA and apelin in diabetic kidney disease may involve slightly different signalling pathways.

Contrast-agents are commonly used in angiographic procedures and are well established as the principal cause of acute kidney injury (Mohammed et al., 2013). Recently, the levels of ELA in coronary angiography patients exposed to iodinated contrast agents was significantly lower compared to pre-exposure levels without a cumulative change in creatinine level. Treatment of tubular epithelial cells in vitro with ELA protected them from iodinated contrast agent-induced apoptosis, oxidative stress and inflammation (Li et al., 2020c). This provides further support for the potential renoprotective role of ELA-apelin receptor signalling pathway in kidney diseases.

### 1.3.1.4 Other Diseases

The role of ELA in many other diseases has not yet been explored. However, several studies have found a potential role for ELA-apelin receptor signalling in different types of cancer. A recent study showed that ELA expression was upregulated in glioblastoma and that this was associated with poor prognosis, implicating the peptide as a potential biomarker for the disease (Artas et al., 2018; Ganguly et al., 2019). In support, serum ELA levels were also found to be increased in patients with chronic lymphocytic leukaemia compared to healthy controls (Acik et al., 2019). Additionally, ELA was overexpressed in various ovarian cancer histotypes, particularly ovarian clear cell carcinoma. In this tumour type, ELA exhibited pro-tumourigenic roles by increasing migration and proliferation in a p53-dependent manner which was blocked by deletion of apela gene (Yi et al., 2017). Interestingly, Yi et al., (2017) claimed that these cells (OVISE) do not express the apelin receptor, suggesting that the effects were independent of the apelin receptor and that ELA may bind another unidentified receptor in this cell type.

A recent study analysed apelin, ELA and apelin receptor expression data on The Cancer Genome Atlas (TCGA). Although ELA expression was upregulated in several
cancers including colon, lung, stomach and thymoma, it was downregulated in all three renal cancer subtypes (renal cell carcinoma chromophobe, papillary and clear cell) (Soulet et al., 2020). However, apelin and apelin receptor were either upregulated or unaffected in these renal cancer subtypes. In addition, ELA gene expression in renal cancer was negatively correlated with proliferation in all renal carcinoma cell types, although apelin and apelin receptor expression showed a positive correlation (Soulet et al., 2020). This, therefore, suggested that exogenous ELA might have therapeutic benefit in renal cancer. Indeed, replacement of the downregulated ELA both \textit{in vitro} and \textit{in vivo} in models of this cancer revealed a tumour suppressor effect via activation of mTORC1/S6K/4EBP1 and Akt/ERK signalling to cause decreased proliferation, metastasis and increased apoptosis (Soulet et al., 2020). Additionally, they showed that combined ELA and sunitinib treatment had a synergistic effect of suppressing tumour growth and angiogenesis in an allograft model (Soulet et al., 2020).

1.4 Synthetic Ligands of the Apelin Receptor

1.4.1 Agonists

The identification of the apelin receptor as a novel therapeutic target in various diseases has led to the development of synthetic ligands with the aim of increasing half-life while preserving efficacy, owing to the short half-life of the endogenous peptides (Davenport et al., 2020). Current strategies have focused on apelin, especially the shorter isoforms. Modifications and analogues of ELA are only beginning to emerge. Principally, modifications of apelin have employed techniques such as polyethylene glycol (PEG)-ylation (Murza et al., 2012), the addition of unnatural amino acids (Juhl et al., 2016; Murza et al., 2015; Wang et al., 2016), cyclisation (Brame et al., 2015; Hamada et al., 2008; Macaluso and Glen, 2010) and acylation (O’Harte et al., 2017, 2018) (Table 1.1). Other approaches such as the identification of a small molecule (non-peptide) agonist (Myers et al., 2020; Read et al., 2016), immunoglobulin Fc-fusion proteins and single-domain antibodies have emerged (Ma et al., 2020).
<table>
<thead>
<tr>
<th>Ligand</th>
<th>Molecule type</th>
<th>Action</th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>pK&lt;sub&gt;i&lt;/sub&gt;</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM07</td>
<td>Peptide</td>
<td>Biased agonist</td>
<td>9.5</td>
<td>-</td>
<td>2-fold less potent agonist activity in β-arrestin; no cAMP data; vasodilation, inotropic actions</td>
<td>(Brame et al., 2015; Yang et al., 2019)</td>
</tr>
<tr>
<td>AlbudAb-MM202</td>
<td>Ab-conjugated peptide</td>
<td>Full agonist</td>
<td>-</td>
<td>9.4</td>
<td>Potent agonist activity in β-arrestin and cAMP; vasodilation, inotropic actions</td>
<td>(Read et al., 2019b)</td>
</tr>
<tr>
<td>JN241-9</td>
<td>Single-domain Ab</td>
<td>Agonist</td>
<td>7.4</td>
<td>-</td>
<td>Inhibited cAMP accumulation, β-arrestin recruitment; vasodilatory and inotropic effect not reported</td>
<td>(Ma et al., 2020)</td>
</tr>
<tr>
<td>AMG 986*</td>
<td>Small molecule</td>
<td>Full agonist</td>
<td>9.6</td>
<td>-</td>
<td>Inhibited cAMP accumulation, β-arrestin recruitment, inotropic actions</td>
<td>(Ason et al., 2020)</td>
</tr>
<tr>
<td>AM8123*</td>
<td>Small molecule</td>
<td>Full agonist</td>
<td>9.4</td>
<td>-</td>
<td>Inhibited cAMP accumulation, β-</td>
<td>(Ason et al., 2020)</td>
</tr>
</tbody>
</table>
Modification of drugs by covalent conjugation of PEG molecules is commonly used to enhance the pharmaceutical properties of drugs and has led to 12 FDA-approved medications since 1990 (Turecek et al., 2016). For peptides, the main benefit of PEGylation has been an extension of plasma half-life, and this has been attempted for apelin peptides. PEGylation of apelin-12 and apelin-17 resulted in the loss of binding to the apelin receptor; however, N-terminal PEGylation of apelin-36 with 40-kDa PEG was well tolerated and did not impair binding affinity (Jia et al., 2012). Infusion of [40-kDa-PEG]-apelin-36 for 20 mins in normal rats showed potent cardiovascular effects (positive inotropy), similar to endogenous apelin-36 but which lasted significantly longer (100 mins) than that of the endogenous peptide (30 mins). This supported prolonged plasma half-life of the PEGylated peptide. A subsequent study identified other regions in apelin-13 that could tolerate PEGylation and showed that it was also able to extend plasma half-life (Murza et al., 2012). Other peptide-based apelin receptor agonists have been developed including CLR325 (Novartis) which has just completed phase two clinical trial for the treatment of heart failure with reduced ejection fraction and diabetes (Pinilla-Vera et al., 2019).

Another technique that has been widely used to extend plasma half-life is the use of domain antibody with high specificity for serum albumin (AlbudAb) and have been successfully used to increase the stability of labile peptides like exendin-4 and GLP-1 (Bao et al., 2013; O’Connor-Semmes et al., 2014). Read et al., (2019b) used this

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| CMF-019* | Small molecule | Full agonist | - | 8.6 | Vasodilation, inotropic actions | (Read et al., 2016) |

*antibody-biased agonists,*small molecule agonists, MM07, is peptide agonist. $pK_i$, negative log of $K_i$ (inhibition constant); $Ab$, antibody; $pEC_{50}$, negative log of $EC_{50}$ (concentration of agonist required to produce half-maximal response)
approach to extend the half-life of apelin-13 analogue MM202 and showed that MM202-AlbudAb retained binding affinity at the human apelin receptor. In normal rats, MM202-AlbudAb induced strong positive inotropic effects resulting in increased cardiac output and stroke volume and decreased mean arterial pressure similar to the endogenous apelin peptide. Recently, using a single-domain antibody-based strategy, a novel apelin receptor agonist, JN241-9 was discovered and shown to have a high affinity for the receptor in vitro (Ma et al., 2020). Overall, these studies suggest that antibody-based agonist discovery could lead to the identification of clinically relevant apelin receptor ligands for treatment of various diseases.

1.5 Aims and Hypothesis

The apelinergic system has crucial roles in the regulation of physiological systems and is dysregulated in disease states, particularly cardio-renal diseases, suggesting that targeting the apelin pathway might be a novel therapeutic strategy for treating these diseases. Although both apelin and apelin receptor are more widely expressed, recent studies suggested that ELA was exclusively expressed in the kidney (Deng et al., 2015; Wang et al., 2015), as can been seen in this data from human protein atlas obtained in 2017 (Fig. 1.6). However, earlier studies indicated that ELA was required for the development of the heart, not kidney, suggesting that ELA may be expressed beyond the kidney. Additionally, it remains unclear why two ligands activate the same receptor. The expression and cellular distribution of the apelin pathway in the kidney are ill-defined. Therefore the working hypothesis of this thesis was that like apelin, ELA was widely expressed in other tissues and that determining the precise anatomical and cellular distribution of apelin/ELA and their target receptor in human kidney will give initial insight in the physiological/pathophysiological role. To address this hypothesis, I have explored three aspects of the apelin signalling pathway.
Firstly, in chapter 3, the expression of ELA relative to apelin and apelin receptor in mouse and human tissues were investigated at the mRNA level. Since the cellular distribution of the apelin system in the kidney is not known, the precise cellular localisation of ELA, apelin and the apelin receptor in different segments of the renal nephron was explored using the therapeutically relevant human tissue. The clinical relevance of the apelin pathway in kidney disease was further investigated using a mouse model of CKD and by examining changes, if any, in the circulating levels of apelin and ELA in CKD patients compared to healthy individuals. Also, using mouse deficient for ELA, the interaction of ELA, apelin and their common apelin receptor was further explored by investigating changes, if any, in apelin and apelin receptor level. The effect of ELA deficiency on the expression of components of the RAS pathway,
ACE and ACE2, known to interact with apelin receptor signalling was also investigated.

The second aim of this study was to investigate the metabolism of mature ELA peptide, ELA-32 \textit{in vitro} in human plasma and kidney homogenates to determine if the shorter isoforms, ELA-21 and ELA-11 were generated from the mature peptide enzymatically as previously suggested (Chapter 4). Using very sensitive mass spectrometers, I sought to identify which ELA isoform was abundantly produced endogenously in human tissues. Additionally, the metabolism of apelin peptides \textit{in vitro} is well-studied, but it is not known which enzymes cleave the peptide or how it was metabolised \textit{in vivo} in humans. In chapter 5 of this thesis, the \textit{in vivo} metabolism of ELA-32 in humans was investigated. This information is essential for the synthesis of pharmacologically and clinically relevant apelin and ELA peptides which are resistant to proteolytic degradation, and thus have prolonged half-life.

Recently, the apelin-36 analogues N-58 (where Ala$^{28}$ substituted Leu$^{28}$) and N-140 (Leu$^{28}$ was replaced with Cys$^{28}$ and PEGylated) was developed to increase plasma half-life and were shown to retain metabolic effects in mice but lost their cardiovascular effects compared to the endogenous apelin-36 (Galon-Tilleman et al., 2017). The authors concluded that the activity of these analogues was independent of the apelin receptor, suggesting that another yet unidentified receptor might transmit apelin signals. Therefore, the final aim of this study was to show that these apelin-36 analogues were indeed biased apelin receptor ligands (chapter 6).
Chapter 2  Materials and Methods

2.1 Materials

[Py1]apelin-13 was custom synthesised by Severn Biotech (Kidderminster, England), purified by HPLC and analysed by mass spectrometry. Peptide (purity >95%) was dispensed under sterile conditions. [Glp65,Nle75,Tyr77][125]apelin-13 (Cat No.: NEX393), was from Perkin Elmer (MA, USA). ELA-11 (Cat No.: 007-22), ELA-21 (Cat. No.: 007-20) and ELA-32 (Cat. No.: 007-19) peptides were from Phoenix Pharmaceuticals, Ltd (Belmont, USA). Stable isotope-labelled [Py1]apelin-13 (pGlu-R-[U-13C5,15N-Pro]-R-[U-13C6,15N-Leu]-SHKGPMPF-acid) and ELA-32 (Ac-QRP-V-NLT-M-RRKLRKHN-C’-LQRR-C’-MPLHSR-[U-13C5,15N-Pro]-[U-13C9,15N-Phe]-P-acid) used as an internal standard were custom synthesised by Cambridge Research Biochemicals (Billingham, England). BioMax MR-1 film, fixer and developer were purchased from Sigma-Aldrich (Gillingham, UK). PureLink™ RNA Mini Kit and PureLink DNase mixture were from Life Technologies (Carlsbad, USA). Wizard SV Gel and PCR Clean-up system were purchased from Promega (Madison, USA). PathHunter® eXpress β-arrestin GPCR assay (AGTRL1) and cAMP assay kits were from DiscoverX (Fremont, USA). Apelin-36 analogues were obtained from AstraZeneca Plc, UK. Protein assay reagents were obtained from Bio-Rad (USA, Cat. No.: 500-0116). Protein LoBind Eppendorf tubes (Cat No.: 0030108094) and 1 ml protein LoBind 96-well plate (Cat No.: 0030504216) were purchased from Eppendorf (Stevenage, UK), and Oasis HLB Prime μ-Elution 96-well plates (Cat No.: 186008052) were purchased from Waters (Waters, Wilmslow, UK). Acetonitrile (ACN) (Cat. No.: 33209-1L) were purchased from Sigma Aldrich (Saint Louis, USA), methanol (Cat. No.: 10675112) and 0.1% formic acid (FA) in water (%v/v) (Cat. No.: LS118-212) were purchased from Fisher Scientific (New Hampshire, USA). Acquity UPLC HSS T3 1.8 μm 2.1x50 mm column (Cat No.: 186003538) used for the LC-MS/MS analysis was obtained from Waters (Wilmslow, UK). Protease inhibitor cocktail (Cat No.: P8340) that inhibits a broad range of
proteases including serine, cysteine and acid proteases, and aminopeptidases, was purchased from Sigma-Aldrich (Gillingham, UK). All other reagents were obtained from Sigma-Aldrich (Gillingham, UK), now Merck.

2.2 Tissue Collection

Human tissues were obtained with informed consent and ethical approval (05/Q104/142) from the Papworth Hospital Research Tissue Bank (08/H0304/56) in conformity with the principles outlined in the declaration of Helsinki. Some kidney samples were dissected into renal cortex and medulla. All tissues were frozen and stored at -70 °C until used. Human left, and right ventricle was from explanted donor hearts for which there was no suitable recipient or heart tissues from dilated cardiomyopathy patients. Human coronary artery was obtained from dilated cardiomyopathy patients receiving heart transplantation. Histologically normal lungs were obtained from patients undergoing lobectomy. Human brain and glioblastoma (GBM) were obtained from the Department of Neurosurgery, University of Cambridge. The histologically normal human kidney was collected from kidney cancer patients undergoing surgery.

The University of Cambridge Animal Welfare and Ethical Review Body (AWERB) regulated all animal research under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review. Female wild type CD-1 mice (30-34g, ~ 5 weeks old) were obtained from Charles River (UK) and housed for additional four weeks until they were ≥12 weeks old before animals were sacrificed using CO₂ overdose followed by cranial dislocation for tissue collection. Additionally, apela (ELA) knockout and heterozygous mice (male and female) were obtained from Dr Anna-Katerina Hadjantonakis (Sloan Kettering Institute, NY, USA). These mice were generated as previously described (Freyer et al., 2017), and tissues made available for the studies described below. Briefly, CRISPR/Cas9 was used to knock-in H2B-GFP-pA reporter cassette bearing Neomycin into the endogenous translational start site at exon 2 of mouse Apela gene using mouse embryonic stem cells (ESCs).
The guide sequence was subcloned into pX335 plasmid, and correctly, targeted mESCs were injected into C57BL/6 blastocysts to generate chimaeras (Freyer et al. 2017). The resulting male chimaeras were crossed to CD-1 females for two generations. In some cases, Neomycin was genetically excised by crossing ApelaH2B-GFP-NEO/+ mice with CAG-Cre mice (Fig. 2.1). The resulting mouse without Neomycin was called NEO-OUT while a knock-in reporter with Neomycin was called NEO-IN.

![Diagram](image)

**Figure 2.1. Targeting strategy for generation of apela (ELA) KO mice. Homology directed repair was used to delete 145 base pairs from exon 2 of the endogenous apela gene in the wild type locus (adapted from (Freyer et al. 2017)).**

### 2.3 RNA Extraction and Expression in Tissues

#### 2.3.1 RNA Extraction

Total RNA was extracted from ~200 mg of human and mouse tissues using the PureLink RNA Mini Kit (RNA Ambion, Life Technologies) according to manufacturer
instructions. The tissues used include human kidney medulla (n=6) and cortex (n=6), brain (n=9), lungs (n=9), left ventricle (n=9), right ventricle (n=9), and WT (n=6), apela heterozygous (apela+/n=8) and apela KO (apela-/- n=8) mouse brain, kidney, heart, spleen and liver. Briefly, each tissue was chopped into tiny pieces with a sterile blade and transferred to a lysing matrix D tube (MP Biomedicals, USA) with 1 ml TRIzol. Tissues were homogenised using the FastPrep-24TM 5G system (MP Biomedicals, USA) for up to 3 runs at 6.5 m/s for 45 secs; incubated (5 mins, room temperature) and chloroform (200 µl) added. Homogenates were mixed by vigorous shaking (15 secs), incubated (3 mins, room temperature) and centrifuged (12,000 xg, 15 mins, 4 °C). The resulting supernatant was then transferred to fresh tubes; mixed with an equal volume of freshly prepared 70% ethanol and transferred to a spin cartridge. The cartridge was spun (12,000 xg, 15 secs, room temperature) to remove TRIzol and chloroform. Genomic DNA was digested by incubating with PureLink DNAse mixture (containing 8 µl 10x DNAse I reaction buffer, 10 µl DNAse (~3 U/µl) and 62 µl DNAsase-free water) for 15 mins. After three more washes, the spin column was dried to evaporate residual ethanol by centrifugation (12,000 xg, 1 min, room temperature) and final RNA eluted. Extracted RNA yield was quantified using NanoDrop 1000 spectrophotometer (Wilmington, USA), and stored at -70°C until needed.

2.3.2 cDNA Synthesis and Purification

First-strand cDNA was synthesised using the Promega Reverse Transcription System kit (A3500) according to manufacturer protocol. Briefly, total RNA (1 µg) was incubated with Oligo dT and Random primers (1 µl each) made up to 10.9 µl with RNase-free water and denatured at 70 °C for 10 mins before cooling at 4 °C for 5 mins on a ProFlex™ PCR System (Applied Biosystems). After adding the reverse transcription mix (comprising 4 µl MgCl₂, 2 µl 10x reverse transcription buffer, 2 µl dNTP, 0.5 µl RNAsin ribonuclease inhibitor & 0.6 µl AMV Reverse Transcriptase), first-strand cDNA synthesis was performed under these conditions: 22 °C for 5 mins, 40 °C for 1 hr, 95 °C for 5 min and 4 °C for 5 mins. In some cases, the resulting cDNA was purified using
the Promega Wizard SV Gel and PCR Clean-Up System kit (Cat. No.: A9282) according to manufacturer protocol.

2.3.3 Quantitative Real-time Polymerase Chain Reaction (qPCR)

To study the mRNA expression of APELA, APLN or APLNR in mouse and human tissues, qPCR was performed for 45 cycles using the ABI 7500 Real-Time PCR System (Life Technologies) with double-dye Taqman primer probes. The mRNA expression of Ace and Ace2 in mouse tissues were also investigated. The Taqman gene expression system comprises a pair of specific PCR primer probes targeting a specific gene of interest, a probe labelled with a fluorescent reporter dye, FAM or VIC, at the 5’ end and a quencher dye at the 3’ end, as well as a Taq DNA polymerase. In this reaction system, the initial high temperature used to denature the double-stranded cDNA allows the quencher molecule to quench the signals from the fluorescent dye at the 5’ end of the probe as long as they are still attached to it. This is followed by a lowered annealing temperature, which enables the primers and Taq polymerase to bind the sequence of the target gene. Taq DNA polymerase synthesises new strands from the primer and the template sequence of the target gene until it reaches a Taqman probe, where its endogenous 5’ exonuclease activity cleaves the probe thereby separating the dye from the quencher. With each PCR amplification cycle, more dye molecules are released, resulting in increased fluorescent intensity that is proportional to the amount of amplicon synthesised. This increase in fluorescence after each cycle is monitored by the instrument and recorded.

In this experiment, cDNA (3.75 μl) was added to 11.25 μl of qPCR master mix (comprising Applied Biosystems TaqMan Gene Expression Master Mix; APELA, APLN or APLNR double-dye primer-probe, and water in the ratio of 10:1:4) per well of DNase-/RNAse-free MicroAmp 96-well plates on ice. The reaction was performed in triplicates for each gene. Human 18S rRNA (Life Technologies) or mouse β-actin (Life Technologies) was used as an internal control for human and mouse samples, respectively. The primer sequence or IDs are shown in Table 2.1. The expression of
APELA, APLN and APLNR, were normalised to 18S or β-actin using the comparative C_T method as previously described (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

Table 2.1. Primer sequences or IDs for RT-qPCR experiments

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence or ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human APELA (Primer design)</td>
<td>Sense GAAGAAGAAGGAGTGAAGGA &lt;br&gt; Antisense CCATTCCAGGTGCTTTCAAT</td>
</tr>
<tr>
<td></td>
<td>Primers from ThermoFisher</td>
</tr>
<tr>
<td>Human 18S rRNA</td>
<td>Hs99999901_s1</td>
</tr>
<tr>
<td>Human APLN</td>
<td>Hs00175572_m1</td>
</tr>
<tr>
<td>Human APLNR</td>
<td>Hs00270873_s1</td>
</tr>
<tr>
<td>Mouse β-actin</td>
<td>Mm00607939_s1</td>
</tr>
<tr>
<td>Mouse Apela</td>
<td>Mm04278373_m1</td>
</tr>
<tr>
<td>Mouse ApIn</td>
<td>Mm00443562_m1</td>
</tr>
<tr>
<td>Mouse ApInr</td>
<td>Mm00442191_s1</td>
</tr>
<tr>
<td>Mouse Ace1</td>
<td>Mm00802048_m1</td>
</tr>
<tr>
<td>Mouse Ace2</td>
<td>Mm01159006_m1</td>
</tr>
</tbody>
</table>

2.4 Sectioning of Tissues

Tissues were sectioned using a cryostat with a motorised microtome (Bright Instrument Co. Ltd, Cambridgeshire, UK). Frozen tissues were mounted on metal
chucks using OCT embedding media and left on the cryostat chamber at -20 °C for about 30-60 mins to set. Tissues were sectioned at a thickness of 15 µm and thaw-mounted onto super frost plus slides; allowed to air dry at room temperature and kept at -70 °C until required.

2.4.1 Immunofluorescence Double Staining

To precisely identify the cellular distribution of apelin, ELA and apelin receptor along the renal nephron in human kidney, dual-label immunofluorescence was performed on fresh-frozen human kidney sections using specific markers (Fig. 2.2). Sections were air-dried overnight and fixed in acetone for 10 mins at room temperature. Slides were ringed with a hydrophobic pen and incubated with 5% goat serum in 1x PBS for 2 hrs at room temperature to block non-specific binding. Primary antibodies were prepared in PBST (PBS and 0.01% Tween 20) containing 3% goat serum and 250 μl of PBST solution containing antibodies added to each slide. All the antibodies used and their concentrations were shown in table 2.2. SGLT2 was only tested with the apelin receptor to determine whether the receptor was colocalised with this protein, given its importance in diabetes treatment (Gallo et al., 2015). Buffer (PBST containing 3% goat serum) instead of the primary antibody was added to negative control sections before all slides were incubated with antibodies overnight at 4 °C. Following three washes in ice-cold PBST, Alexa Fluor 488 goat anti-rabbit IgG, Hoechst (1:100) and Alexa Fluor 568 goat anti-mouse IgG in buffer were added and incubated for 1 hr at room temperature. Sections were washed 3x 5mins in PBST and mounted with ProLong Gold Antifade reagent. Slides were imaged using Zeiss 510 Meta confocal laser scanning microscope (Heidelberg, Germany) or Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems, Milton Keynes, UK) at x40 magnification. Images were processed using Fiji (ImageJ) for background subtraction using the rolling ball method before merging channels.
Table 2.2. List of antibodies used showing the company, immunogen, concentration used and species.

<table>
<thead>
<tr>
<th>Protein (human)</th>
<th>Verification</th>
<th>Species</th>
<th>Immunogen</th>
<th>Concentration used</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apelin</td>
<td>9 literature references</td>
<td>Rabbit</td>
<td>C-terminal</td>
<td>1:50</td>
<td>Abcam</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Cat No.: ab59469)</td>
</tr>
<tr>
<td>Apelin receptor</td>
<td>8 literature references</td>
<td>Rabbit</td>
<td>C-terminal</td>
<td>1:100</td>
<td>Abcam</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Cat No.: ab84296)</td>
</tr>
<tr>
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<td>Unknown</td>
<td>1:50</td>
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<td></td>
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<td>(Cat. No.: M061601-2)</td>
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<tr>
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<td>Santa Cruz</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Aquaporin 4 (AQP4)</td>
<td>6 literature references</td>
<td>Mouse</td>
<td>Unknown</td>
<td>1:100</td>
<td>Abcam</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Cat No.: ab9512)</td>
</tr>
<tr>
<td>Sodium potassium chloride cotransporter (NKCC2)</td>
<td>-</td>
<td>Mouse</td>
<td>Unknown</td>
<td>1:100</td>
<td>Santa Cruz</td>
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</tr>
<tr>
<td>Antibody Description</td>
<td>Literature References</td>
<td>Species</td>
<td>Isotype</td>
<td>Dilution</td>
<td>Vendor</td>
</tr>
<tr>
<td>----------------------------------------------------------</td>
<td>------------------------</td>
<td>---------</td>
<td>---------------</td>
<td>----------</td>
<td>-----------------------</td>
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<tr>
<td>Transient receptor potential melastatin 6 (TRPM6)</td>
<td>2</td>
<td>Mouse</td>
<td>Unknown</td>
<td>1:100</td>
<td>Santa Cruz (Cat No.: sc-365536)</td>
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<tr>
<td>Sodium-glucose cotransporter 2 (SGLT2)</td>
<td>10</td>
<td>Mouse</td>
<td>Unknown</td>
<td>1:50</td>
<td>Santa Cruz (Cat No.: sc-393350)</td>
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Secondary antibodies

<table>
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<tr>
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<th>Species</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Goat</td>
<td>Rabbit IgG</td>
<td>1:200</td>
<td>Invitrogen (Cat. No.: 11034)</td>
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<tr>
<td>Alexa Fluor 568 goat anti-mouse IgG</td>
<td>264</td>
<td>Goat</td>
<td>Mouse IgG</td>
<td>1:200</td>
<td>Invitrogen (Cat No.: 11031)</td>
</tr>
</tbody>
</table>

*No literature reference but manufacturer references available; †this antibody has now been discontinued by the manufacturer; ‡this antibody was further verified in knockout mice*
Figure 2.2. Anatomy of the renal nephron. PCT, proximal convoluted tubule (PCT), PCT S3 represent segment 3, also called proximal straight tubule (PST); TDL, the thin descending limb of the loop of Henle; TAL, thick ascending limb of the loop of Henle; DCT, distal convoluted tubule; and CD, collecting duct (modified from Gamba (2005)).
To verify the localisation of the apelin system in kidney tubular cells, 4% formaldehyde-fixed paraffin wax-embedded 5 µm thick kidney sections were a gift from Prof Fiona Karet, University of Cambridge. The slides were loaded onto slide holders and placed in Histoclear I and II for 10 mins each at room temperature. Slides were then incubated for 5 mins each in graded methanol series (100% methanol 1, 100% methanol 2, 90% methanol, 70% methanol and water, respectively) and transferred to citrate antigen retrieval buffer (pH 6) in 2100 Antigen Retriever (BioVendor GmbH, Kassel, Germany) for 2 hrs according to manufacturer instructions. Sections were subsequently blocked in 5% goat serum in PBS for 2 hrs and incubated with primary antibodies (Rabbit polyclonal anti-apelin receptor, apelin and ELA-32 antibody and monoclonal mouse anti-aquaporin 1 antibody) at the concentrations state in table 2.2 overnight at 4 °C before secondary antibody was added after 3x 5 mins washes. Slides were mounted with ProLong Gold Antifade reagent and imaged using Zeiss 510 Meta confocal laser scanning microscope (Heidelberg, Germany) at x40 magnification. Images were processed using Fiji (ImageJ) for background subtraction using the rolling ball method before merging channels.

2.4.2 Protein Extraction and Quantification

2.4.2.1 Homogenate Preparation

Human left ventricle used for homogenate preparation was obtained from five dilated cardiomyopathy patients (male and female aged 38-55 years). Left ventricle tissues were pooled together and 2 g tissue transferred into a centrifuge tube (50 ml) containing 20 ml homogenisation buffer (50 mM Tris, 5 mM MgCl$_2$, 5 mM EDTA, 1 mM EGTA at pH 7.4 with protease inhibitor cocktail) on ice. The tissue was homogenised using a polytron probe with 2-3x 20 secs bursts on ice and centrifuged (1,000 xg, 2 mins, 4 °C). The supernatant was transferred to new tubes on ice and centrifuged (40,910xg, 30 mins, 4 °C). The resulting pellet was resuspended in 5 ml homogenisation buffer containing protease inhibitor cocktail and centrifuged (40,910 xg, 30 mins, 4 °C). Pellets were then resuspended in 10 ml HEPES buffer (50 mM
HEPES, pH 7.4) and protein concentration determined using the Bio-Rad DC Protein Assay. Samples were stored at -70 °C until required.

To extract protein from rat heart, hearts collected from adult male and female Sprague Dawley rats (n = 14; aged 7-10 weeks) were pooled together, and 2 g heart tissue transferred to a centrifuge tube and homogenates prepared as described above.

Protein was also extracted from the human kidney (n=3) as described above and used for saturation binding experiments.

2.4.2.2 Protein Assay

Protein concentration was determined using Bio-Rad DC Protein Assay (Bio-Rad, Cat. No.: 500-0116), a colourimetric assay similar to the Lowry Assay. This assay is based on the reaction of proteins with an alkaline copper tartrate solution and Folin reagent in two steps. The first step involving the reaction between protein and copper in an alkaline medium followed by reduction of Folin reagent by the copper-treated protein resulting in the production of characteristic blue colour with absorbance minima and maxima at 405 nm and 750 nm respectively. In this assay, 3.75 mg/ml BSA was prepared in solubilisation buffer (0.5 M NaOH, 1% SDS) and diluted to a final BSA standard concentration range of 0.1875-1.5 mg/ml in the same buffer. The homogenate sample (100 µl) was then solubilised by mixing with 100 µl solubilisation buffer, before incubation for 30 mins at 80 °C. Samples were subsequently centrifuged (11,000 xg, 5 mins, room temperature) before adding 5 µl of the supernatant, blank or BSA standard to empty wells of a 96-well plate. A 25 µl aliquot of Reagent A’ comprising 2.5 ml reagent A and 50 µl reagent S per plate was added to each well before adding 200 µl/well of reagent B and incubating for 5 mins at room temperature. Absorbance at 750 nm was read using an EL800 Universal Microplate Reader (BioTek Instruments).
2.5 Receptor Binding Experiments

Receptor binding experiments were performed to quantify the affinity of [Pyr₁]apelin-13 for the apelin receptor and to measure the density of the apelin receptor using [¹²⁵I]apelin-13 alone or in the presence of cold ligand ([Pyr₁]apelin-13) as previously described (Davenport and Kuc, 2005). All tubes and pipette tips used for these experiments were treated with Sigmacote (Sigma-Aldrich, Gillingham, UK) to reduce non-specific binding of the radiolabel to plastic consumables.

2.5.1 Saturation Binding Experiment

Protein homogenates were diluted to 4.5 mg/ml (1.5 mg/ml final concentration) in assay buffer (Tris 50 mM, MgCl₂ 5 mM, pH 7.4). A two-fold serial dilution of [¹²⁵I]apelin-13 was performed in assay buffer to achieve a final concentration range of 2 nM - 3.9 pM in sigmacoted tubes. Protein samples (100 µl) were incubated with increasing concentrations of [¹²⁵I]apelin-13 (100 µl) in the absence (total binding) or presence of 2 µM [Pyr₁]apelin-13 (non-specific binding), for 90 mins at 4 °C, until equilibrium was reached and centrifuged (20,000 xg, 10 mins, 4 °C) to terminate the reaction. The resulting pellets were washed with 500 µl ice-cold wash buffer (Tris-HCl buffer; 50 mM Tris, pH 7.4) before second centrifugation at the same settings. Pellets and solutions of total and non-specific binding were counted on a Cobra II Auto-Gamma radiation counter (Packard) to obtain radioactivity in disintegrations per minute (DPM). This was used in the KELL program to determine the concentration of [¹²⁵I]apelin-13 that bind 50% of the apelin receptors (Kᵤ, the equilibrium dissociation constant) and apelin receptor density (Bₘₐₓ) (McPherson, 1985).

Saturation binding data analyses were performed using the non-linear iterative curve-fitting program, RADLIG software (version 6) of the Equilibrium Binding Data Analysis (EBDA) and LIGAND programs (KELL, BIOSOFT, Cambridge, UK) (McPherson, 1985). This program performs an initial analysis of data obtained from saturation studies by converting radioactivity in disintegrations per minute (DPM) to
concentrations using the specific activity of the radiolabel (~2200 Ci/mmol for \([^{125}\text{I}]\text{apelin-13}\), where 1 Ci = 2.22x10^{12} DPM. This conversion factor was used to convert specific activity of the radiolabel to DPM/fmol (4.884x10^3 DPM/fmol), which was further converted to specific binding (fmol/mg protein) using the known protein concentration used in the assay. The Scatchard and Hill analyses performed by EBDA provided initial estimates that were used in the LIGAND component of the KELL Program (Maguire et al., 2012). This component of the program uses weighted, non-linear iterative curve-fitting to derive the final values for receptor density (\(B_{\text{Max}}\)) and \(K_D\). The \(B_{\text{Max}}\) and \(K_D\) were then expressed as mean±SEM.

### 2.5.2 Radioligand Competition Binding Assay

Competition binding experiments were performed on pooled human homogenised left ventricle (HLV, 1.5 mg/ml) and pooled rat heart homogenates (1.5 mg/ml) as previously described (Read et al., 2016; Yang et al., 2017b). Homogenates were incubated for 90 mins with \([^{125}\text{I}]\text{apelin-13}\) (0.1 nM) in assay buffer (Tris 50 mM, MgCl\(_2\) 5 nM, pH 7.4), in the presence of increasing concentrations of apelin-36, apelin-36-[L28A] or apelin-36-[L28C(30kDa-PEG)] (5x10^{-11}-10^{-4} M). Binding in the presence of 2 \(\mu\)M [Pyr\(^1\)]apelin-13 was considered non-specific. The samples were centrifuged (20,000 xg, 10 mins, 4 °C) before pellets were washed with Tris-HCl buffer (50 mM, pH 7.4) on ice to remove any unbound ligands. The samples were then re-centrifuged to collect protein pellets before bound radioactivity in these pellets were counted using Packard Cobra II Auto Gamma Counter (Packard). Data were analysed with Graphpad Prism 6 (Graphpad Software, Inc. La Jolla, CA), using the One-site Fit \(K_i\) equation. The \(K_D\) values (human heart, 0.076 nM; rat heart, 0.28 nM) for the radiolabel were obtained from the saturation binding assays (section 2.7.1) and subsequently used in the GraphPad Prism analysis to determine the \(K_i\) (equilibrium dissociation constant obtained from a competition binding experiment).
2.5.3 Receptor Autoradiography

Human and mouse kidney sections were pre-incubated for 20 mins in binding buffer (50 mM Tris, 5 mM MgCl₂, pH 7.4) at room temperature before incubating for 90 mins with 0.5 nM [¹²⁵I]apelin-13 in binding buffer. Non-specific binding was defined as binding of [¹²⁵I]apelin-13 (0.5 nM) in the presence of 2 µM [Pyr¹]apelin-13. Slides were then washed (3x 5mins) with ice-cold Tris-HCl buffer (50 mM Tris, pH 7.4), before rinsing in ice-cold deionised water. All slides were air-dried at room temperature before being exposed to emulsion-coated film (Kodak, USA) for 1-2 weeks at room temperature in the dark. Films were developed in 20% v/v developer for 3-5 mins with rocking, rinsed in deionised water for 1 min before incubating for 10 mins in 20% v/v fixer with constant agitation in the dark. The film was washed in running water for 15 mins, air-dried and imaged using a PixeLINK Camera attached a Wild M3Z microscope (Heerbrugg, Switzerland).

2.6 Cell Signalling Assays

2.6.1 Inhibition of cAMP Accumulation Assay

The DiscoverX cAMP Hunter eXpress GPCR (AGTRL1) assay was used to G protein activity of the apelin receptor. This assay exploits the natural coupling status of GPCRs with second messengers (cAMP in this case) to monitor the activation of Gαᵢ or Gαₛ signalling as a measure of cellular cAMP level based on a competitive immunoassay called Enzyme Fragment Complementation (EFC) technology. EFC utilises a fragment of the β-galactosidase called enzyme donor (ED) conjugated to cAMP, which competes with cellular cAMP for binding to an anti-cAMP antibody, and another fragment of the enzyme called Enzyme Acceptor (EA). Thus, at lower cellular cAMP levels all the ED-tagged cAMP binds to an anti-cAMP antibody and are therefore unable to complement the EA resulting in no chemiluminescent signal. However, in the presence of high cellular cAMP levels, the anti-cAMP antibody is saturated, allowing ED-cAMP complex to complement the β-galactosidase (EA), thereby forming an active enzyme. This enzyme hydrolyses a substrate to produce a
chemiluminescent signal that is directly proportional to the amount of cAMP in the cells. For Gαi-coupled GPCRs like the apelin receptor, cAMP production is stimulated using forskolin concurrently with an apelin receptor ligand to monitor the inhibition of cAMP production.

The cAMP assay was performed as previously described (Read et al., 2016; Yang et al., 2017b). Briefly, Chinese Hamster Ovary cells (CHO-K1) cells artificially expressing the human apelin receptor were seeded in Cell Plating media into 96-well plates and incubated for 24 hrs at 37 °C in 5% CO₂. The media was replaced with cAMP Antibody Reagent in Cell Assay Buffer before adding 15 µM forskolin in the presence of human [Pyr¹]apelin-13, human apelin-36, apelin-36-[F36A], apelin-36-[L28A], apelin-36-[L28C(30kDa-PEG)], apelin-36-[A13 A28] and [40kDa-PEG]-apelin-36 (1 pM-0.3 µM), followed by 30 mins incubation at 37 °C in 5% CO₂. Cells were subsequently incubated with cAMP Detection Reagent comprising cAMP Lysis Buffer, Substrate Reagent 1, Substrate Reagent 2 and cAMP Solution D for 1 hr at room temperature followed by 3 hrs incubation with cAMP Reagent A at room temperature and chemiluminescence reading (LumiLITE™ Microplate Reader, DiscoverRx). Responses were fitted to a 4-parameter logistic equation in GraphPad Prism 6 (La Jolla, CA, USA) and values of potency, pD₂ (-log₁₀ EC₅₀, where EC₅₀ is the concentration producing half-maximal response) determined. Data were expressed as percentage inhibition of forskolin-stimulated cAMP production.

2.6.2 β-Arrestin Recruitment Assay

β-Arrestin recruitment experiments were performed using the DiscoveRx PathHunter® β-Arrestin GPCR (AGTRL1) Assay (DiscoverX, Lot No.: 16L0902). Similar to the cAMP assay described above, this assay also uses the EFC technology based on the ability of GPCRs to recruit β-arrestin following ligand binding (Fig. 2.3). In this assay, the apelin receptor is tagged with a fragment of β-galactosidase called ProLink™ (PK) and co-expressed in cells stably expressing β-arrestin conjugated to another fragment of the β-galactosidase called Enzyme Acceptor. Independently these fragments have
no β-galactosidase activity; however, activation of the receptor results in β-arrestin binding to the PK-tagged receptor, thereby forcing the complementation of the two enzyme fragments. This produces an active β-galactosidase enzyme, which hydrolyses a substrate resulting in the production of a chemiluminescent signal that can be used to assess receptor activation directly.

Figure 2.3. Schematic representation of the underlying principle of DiscoveRx β-arrestin assay. In brief, the apelin receptor stably expressed in CHO-K1 cells is tagged with a fragment of β-galactosidase while β-arrestin is tagged with a complementary component. In the absence of ligand, there is no β-galactosidase activity, but upon ligand binding, β-arrestin is recruited, resulting in the activation of β-galactosidase. The enzyme then hydrolyses a substrate in solution to produce a chemiluminescent signal.
Experiments were performed according to methods previously described (Read et al., 2016; Yang et al., 2017b). CHO-K1 cells artificially expressing the human apelin receptor were seeded in Cell Plating media into 96-well plates and incubated for 48 hrs at 37 °C in 5% CO₂. Serial dilutions (30 µM-10 pM final concentration) of human [Pyr¹]apelin-13, human apelin-36, apelin-36-[F36A], apelin-36-[L28A], apelin-36-[L28C(30kDa-PEG)], apelin-36-[A13 A28] and [40kDa-PEG]-apelin-36 were made in Cell Plating media from a 1 mM stock solution (in water). A 5 µl aliquot of apelin-36, apelin-36-[F36A], apelin-36-[L28A], apelin-36-[L28C(30kDa-PEG)], apelin-36-[A13 A28] and [40kDa-PEG]-apelin-36 was used to construct concentration-response curves in duplicate, and plates incubated for 90 mins at 37 °C, in 5% CO₂ before Detection Reagent (27.5 µl/well) was added and further incubated for 2 hr at room temperature in the dark. Negative controls were defined with equal volumes of drug solvent (water). The resulting chemiluminescent signal was measured using a LumiLITE™ Microplate Reader (DiscoverX, Fremont, CA). Responses, measured in relative light units, were fitted with a 4-parameter logistic equation in GraphPad Prism 6 (La Jolla, CA, USA) and values of pD₂ and maximum response (Eₘₐₓ) were calculated for each compound. The data were subsequently normalised to the maximum response to [Pyr¹]apelin-13 used as reference agonist (positive control) in the assay.

2.6.3 Bias Calculations

Bias signalling was determined by comparing the response of apelin-36 analogues in β-arrestin assays to those elicited in the cAMP assay. Bias calculations were performed as previously described using the operational model for bias (van der Westhuizen et al., 2014), and Graphpad prism, to obtain values for logR of the apelin-36 synthetic analogues compared to apelin-36 within each cell-based assay. The logR value is a logarithm of the transduction ratio (τ/Kₐ), where τ is a measure of the coupling efficiency, or efficacy of the agonist and Kₐ is the functional affinity, giving a unique indication of activity for each agonist. The difference between the logR values

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of each analogue and the logR value of the native apelin-36 used as reference peptide was used to determine ΔlogR, that is ΔlogR = mean logR_{analogue} – logR_{apelin-36}. The relative effectiveness defined by the antilog of ΔlogR for each peptide was also determined. Bias factors were calculated from the antilog of ΔΔlogR values, where ΔΔlogR = ΔlogR_{cAMP pathway} - ΔlogR_{β-arrestin}. Thus, the preference of each peptide analogue for the two pathways – β-arrestin (G protein independent) and cAMP (G protein-dependent) were determined.

### 2.7 Analysis of ELA and Apelin Peptides by Mass Spectrometry

In this thesis, two different mass spectrometers were used, namely the triple quadrupole mass spectrometer (TQX-MS) for analyte quantification and Thermofisher Q-Exactive plus orbitrap mass spectrometer (for identification of analytes). The triple quadrupole mass spectrometer uses two mass filters (quadrupoles) separated by a collision cell for collision-induced dissociation (CID) also called ‘quadrupole 2’ to accurately quantify known analytes in a complex matrix (Domon and Aebersold, 2006). Selected/multiple reaction monitoring (SRM/MRM) was performed where only a precursor ion chosen in the first mass filter (quadrupole 1, Q1) was allowed to enter the collision cell (quadrupole 2) for fragmentation before the resulting product ions were filtered by the third quadrupole and sent to the detector (Fig. 2.4). The detector record these signals as a function of chromatographic elution time. This facilitates monitoring of a particular pair of precursor and product ions, called ‘transitions’ with increased selectivity and sensitivity. The advantage of using two levels of mass filter and CID is a significantly reduced overall background noise resulting in greater sensitivity and selectivity of analyte (Domon and Aebersold, 2006). However, this system has very low resolution and mass accuracy and was therefore unsuitable for identification of unknown peptides.
Figure 2.4. Schematic representation of triple quadrupole mass spectrometry selected reaction monitoring principle. In LC-MS/MS, analyte fractions from the high-performance liquid chromatography are ionised by electrospray ionisation. The ionised analytes are delivered into the first quadrupole (Q1) mass filter where a specific precursor ion is selected and sent to the collision cell (q2). The collision cell is a non-mass-resolving radio frequency-only quadrupole used for collision-induced dissociation (CID) to fragment precursor ions into product ions. These product ions are then filtered by the last quadrupole (Q3) and sent to the detector. A series of this kind of reaction can be performed simultaneously on the triple quadrupole mass spectrometer, and the process is called multiple reaction monitoring (MRM).

The orbitrap mass spectrometry used for identification of unknown analytes (peptides) in this study, operates on the principle of separating ions in an oscillating electric field and has very high resolution and mass accuracy (Domon and Aebersold, 2006). This high resolution and mass accuracy made it easier to determine the charge state and mass of multiply charged analytes in complex mixtures (Eliuk and Makarov, 2015) accurately. In this study, these properties were coupled to database searching for identification of ELA peptides in complex tissue extracts.

2.7.1 ELA Analysis by Mass Spectrometry

2.7.1.1 Method Development for Detection of ELA on TQX-MS

To develop an LC-MS/MS method for detection and quantification of endogenous ELA peptides, exogenously synthesised ELA-32, ELA-21 and ELA-11 obtained from Phoenix Pharmaceuticals, Ltd were solubilised in nuclease-free water and run on the
LC-MS/MS. In a subsequent LC-MS/MS analysis after solid-phase extraction, ELA-11 was not detected possibly due to reactivity of the free N-terminal cysteine residues. Hence, further method development was performed with the reduction and alkylation of ELA. All three ELA isoforms; ELA-32, ELA-21 and ELA-11, were mixed to a final concentration of 11 µg/ml. Then 100 µl aliquot of ELA mixture was added to 650 µl 10 mM Dithiothreitol (DTT) in 50 mM ammonium bicarbonate and incubated at 60 °C for 1 hr before adding 150 µl 100 mM iodoacetamide (IAA) in 50 mM ammonium bicarbonate solution and incubated in the dark for 30 mins at room temperature. This was followed by a 30 mins incubation at room temperature in the light before 200 µl 1% formic acid (v/v) was added. This mixture was transferred to small tubes for mass spectrometry analysis.

Samples (10 µl) were injected onto a Waters UPLC H-Class LC system (Waters, Milford, MA) using a 2.1 × 50 mm 1.8 mm particle HSS T3 Acquity column held at 60 °C and flowing at 350 µL/minute. Initial conditions were 92.5% A (0.1% formic acid in water and 7.5% B (0.1% formic acid in ACN), flowing at 350 µL/min at a column temperature of 60 °C. The mobile phase was raised to 40% B over 7 minutes, then flushed at 90%B for 1.5 minutes before returning to initial conditions for a total run time of 10 minutes. The LC eluent was linked to a TQ-XS triple quadrupole (Waters, Milford, MA) running in positive ion mode with a voltage of 3.0 kV, the gas flow of 1000 L/hour, desolvation temperature of 600 °C and a cone voltage of 40 V. Transitions used to monitor for the ELA peptides are outlined in Table 2.3.
Table 2.3. Peptide LC-MS/MS parameters for the SRM analysis of three ELA peptides, including precursor-product transition details and collision cell energy values.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Charge state</th>
<th>Precursor m/z (Q1)</th>
<th>Collision energy eV (Q2)</th>
<th>Product m/z (Q3)</th>
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<tbody>
<tr>
<td>ELA 32 (transition 1)</td>
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<td>581.84</td>
<td>18</td>
<td>659.5</td>
</tr>
<tr>
<td>ELA 32 (transition 2)</td>
<td>7</td>
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<td>654.78</td>
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<tr>
<td>ELA 32 (transition 3)</td>
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<td>581.84</td>
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<td>ELA 21 (transition 1)</td>
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</tr>
<tr>
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<td>6</td>
<td>451.44</td>
<td>13</td>
<td>512.75</td>
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<tr>
<td>ELA 11 (transition 1)</td>
<td>3</td>
<td>447.69</td>
<td>13</td>
<td>613.31</td>
</tr>
<tr>
<td>ELA 11 (transition 2)</td>
<td>3</td>
<td>447.69</td>
<td>13</td>
<td>525.28</td>
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<tr>
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<td>3</td>
<td>447.69</td>
<td>15</td>
<td>115.94</td>
</tr>
</tbody>
</table>

All subsequent ELA analysis on the TQX-MS were done using these SRM transitions and the peak areas for each of the ELA peptides, generated using Quanlynx program (Waters).

2.7.1.2 Peptide Extraction from Human Tissues

Approximately 25 and 50 mg tissue (human kidney, n=5; coronary artery, n=7) were transferred into Lysing MatrixD ceramic beads tubes (MP Bio, 116913100 MP). A 12.5 µl aliquot of a mixture of all three ELA at a concentration of 10 µg/ml and the same amount of ELA-32 internal standard was used to test recovery of peptides. Internal standard (250 ng/ml) in 500 µl of guanidine hydrochloride (6 M) was added to kidney and coronary artery samples and homogenised using the FastPrep-24TM 5G system (MP Biomedicals, USA) for up to 4 runs at 6.0 m/s for 40 seconds. Following
homogenisation, samples were centrifuged (1,000 xg, 5 mins, 4 °C) and 200 µl of the supernatant transferred to protein LoBind tubes. An 800 µl aliquot of 80% (v/v) acetonitrile in deionised water was added to each tube and centrifuged (12,000 xg, 5 mins, 4 °C). The organic phase was discarded and the aqueous (second phase) containing the peptides transferred to a 96-well protein LoBind plate and evaporated under nitrogenised air at 40 °C. Samples were reconstituted in 500 µl 0.1% formic acid in water, vortex-mixed and transferred to 96-well Oasis Primed HLB μ-Elution plate (Waters) and placed under Waters Positive Pressure 96. After washing with 5% methanol, 1% acetic acid in water, peptides were eluted with 2x 30 µl 60% methanol, 10% acetic acid (v/v) in water. The eluates were centrifuged (1,200 rpm, 2 mins, 4 °C) and evaporated under nitrogenised air. Peptides were subsequently reduced and alkylated with 75 µl of 10 mM DTT at 60 °C for 60 mins, 15 µl of 100 mM iodoacetamide (IAA) at room temperature for 30 mins in the dark followed by another 30 mins in the light before 20 µl 1% formic acid was added and stored at -20 °C until analysis on TQX-MS. Generally, the recovery of peptides via this extraction method was very poor, but after analysing extracts on the Orbitrap mass spectrometer without detecting ELA in the samples, a new method for peptide enrichment was sought. Of note, ELA isoforms were detected in positive control samples each time.

### 2.7.1.3 Antibody Coating to Magnetic Beads

Two different magnetic bead system were tested for immunoprecipitation of ELA peptides from tissue homogenates, namely Dynabeads M-280 Tosylactivated (Invitrogen, Cat No.: 14203) and Sera-Mag SpeedBeads Magnetic Protein A/G beads (GE Healthcare, Cat No.: 1715210401150) (Fig. 2.5). The Protein A/G beads combine four IgG binding domains from Protein A (a recombinant *Staphylococcus aureus* cell wall protein) and two IgG-binding domains of Protein G (a recombinant *Streptococci* cell wall protein) to bind antibodies efficiently. Purified [pGlu1]ELA-32 antibody (Cat. No.: H-007-19) which cross-reacts with all three isoforms of ELA or chemerin antibody (used as negative control) or buffer was coated unto Dynabeads according to manufacturer protocols. Briefly, approximately 5 mg (165 µl) beads were
washed with sodium phosphate buffer (0.1 M, pH 7.4) and 100 μg of antibody made up to 150 μl (final volume) with sodium phosphate buffer added. After adding 100 μl of ammonium sulphate buffer (3 M, pH 7.4), beads were incubated at 37°C with end-end rotation (15 m/s) overnight, and unspecific binding blocked with 0.5% BSA in PBS by incubating for 1 hr at 37°C. The beads were then washed twice with PBS containing 0.1% BSA and reconstituted at a concentration of 20 mg/ml in the same buffer.

For Protein A/G beads, antibody coupling was performed according to manufacturer instructions using purified and unpurified [pGlu1]ELA-32 antibody (Phoenix, Cat No.: G-007-19 and H-007-19 respectively) which cross-reacts with all three isoforms of ELA. Briefly, 50 μl of Protein A/G beads were transferred into a 1.5 ml Eppendorf tube and washed using 150 μl wash(binding buffer. After a second wash with 1 ml wash(binding buffer, 10 μl of purified ELA-32 antibody or 5 μl of unpurified ELA-32 antibody was added to the beads in 490 μl of the wash(binding buffer and incubated for 2 hrs at room temperature with end-to-end rotation. The beads were washed with 500 μl of wash(binding buffer to remove unbound antibody before adding the antigen solution.
Figure 2.5. Schematic of the ligand-binding mechanism of magnetic beads used. A, Dynabeads M-280 tosylactivated bind to ligands by covalent bonding; B, Protein A/G sera-magnetic beads. Notice in A, that the antibody coupling occurs at random so antibodies can also bind to beads with their antigen-binding Fab domain, but in B, the antibodies can only bind to the beads with their Fc domain, making their Fab domain free for antigen binding.

2.7.1.4 Validation of ELA Antibody Affinity for the Three ELA Isoforms by Immunoprecipitation

The antibody-coupled beads were incubated with a mixture of all three isoforms of ELA (5 ng/ml and 250 ng/ml) overnight at 4 °C. After washing according to manufacturer’s instructions with wash buffer (protein A/G beads only) or sodium phosphate buffer (0.1 M, pH 7.4) for Dynabeads, peptides were eluted with a solvent
containing 60% methanol and 10% acetic acid in water (100 µl) by incubating with end-to-end rotation for 20 mins at room temperature. The eluate was evaporated, and peptides reduced and alkylated as previously described before 20 µl of the extract was injected into the TQX-MS system for analysis. Compared to Dynabeads, Protein A/G beads immunoprecipitated the highest amount of ELA peptides and was used for all subsequent experiments.

### 2.7.1.5 Peptide Extraction for Immunoprecipitation

Human kidney (193-241 mg), normal brain (~300mg), glioblastoma samples (~300mg) and coronary artery (93-173 mg) samples were transferred into Lysing MatrixD ceramic bead tubes (MP Bio, 116913100 MP) and stored at -80°C until it was needed. Boiling water (1 ml) was added to each tube, as previously described (Pauli et al., 2014). Samples were boiled for 20 mins at 100 °C, cooled to room temperature and homogenised using the FastPrep-24TM 5G system (MP Biomedicals, USA) for up to 3 runs at 6.0 m/s for 40 secs. The resulting homogenate was centrifuged (1,000 xg, 5 mins, 4 °C) and the precipitate transferred to a protein Lobind tube containing antibody-coated beads. A mixture of ELA peptides (all three isoforms) at 100 ng/ml final concentration was added to a tube containing 0.1% BSA and 10 µl ELA-32 antibody (1 mg/ml) as a positive control. All samples were then incubated at 4 °C overnight with end-to-end rotation. Following a 2x 500 µl wash in deionised water, the peptides were eluted by incubating beads with 100 µl of 60% methanol, 10% acetic acid in water (v/v) for 20 mins with end-to-end rotation. In addition, an 8-point calibration line (10 pg/ml-50,000 pg/ml) was generated using 0.1% BSA as surrogate matrix from a stock (10 µg/ml) of ELA-32, ELA-21 and ELA-11 all mixed together. The eluted peptides and 150 µl of each standard were dried down under a stream of oxygen-free nitrogen heated to 40 °C using a Biotage SPE (Upsala, Sweden) evaporation system and reduced and alkylated as previously described. About 20 µl of the extract was injected into ThermoFisher Q-Exactive Orbitrap mass spectrometer for analysis in nanoflow.
2.7.1.6 **Acid-based Extraction**

Tissue homogenate sample (200 µl) was mixed with 0.25% acetic acid, as previously described (Pauli et al., 2014). Also, to increase the chance of detecting all ELA peptides at much higher concentration 500 µl of tissue homogenate was mixed with 250 µl guanidine hydrochloride (6 M) and precipitated with 75% acetonitrile in water (v/v). This was centrifuged (12,000 xg, 5 mins, 4 °C) and the supernatant transferred to a new tube. The precipitate was reconstituted in 0.5% TFA in water (v/v) to ion-pair with any positively ELA peptide that may have remained in the sample and centrifuged (12,000 xg, 5 mins, 4 °C). The supernatant was transferred into a fresh tube and dried down before solid-phase extraction as described above. After SPE, samples were pooled together and dried down before reduction and alkylation. Samples were then analysed on the Orbitrap mass spectrometer in the nanoflow.

2.7.1.7 **ELA Degradation in Human Plasma and Human Kidney Homogenate**

To assess the degradation of ELA-32 in vitro, blood from four healthy human donors (3 male, 1 female) were centrifuged (2,000 xg, 5 mins, 4 °C) and the resulting plasma pooled together. For metabolism in human kidney homogenate, 2 g of human kidney (n=3) was pooled together into 50 mM HEPES solution pH 7.4 and homogenised using a polytron probe with 3x 20 secs burst. Both human plasma and homogenate samples were stored at -70 °C until they were needed. A 1200 µl aliquot of human plasma and 1800 µl aliquot of human tissue homogenate was incubated in a water bath at 37 °C for 25 mins, before blank samples were withdrawn. Degradation was initiated by addition ELA-32 at a final concentration of 5 µg/ml to samples in a water bath at 37 °C. Samples (plasma 50 µl; homogenate 100 µl) were withdrawn at different time-points (plasma: 0, 2, 5, 10, 15, 30, 60, 120, 240; homogenate: 0, 2, 5, 10, 15, 30, 60, 120); and mixed with half volume ice-cold guanidine hydrochloride (6 M) to terminate the reaction. Proteins were precipitated with 300 µl (for plasma) or 500 µl (for kidney homogenate) of 75% ACN in water. Samples were centrifuged (12,000 xg, 5 mins, 4 °C).
°C) and the resulting supernatant transferred to Protein Lobind plate before evaporating on the SPE system described above. Dried residues were reconstituted in 500 µl 0.1% formic acid in water, vortex-mixed and transferred to 96-well Oasis Primed HLB µ-Elution plate (Waters) and placed under Waters Positive Pressure 96. After washing with 5% methanol, 1% acetic acid in water, peptides were eluted with 2x 50 µl 60% methanol, 10% acetic acid (v/v) in water and dried down under nitrogenised air. Extracts were then reduced and alkylated as described above. Samples were analysed on ThermoScientific Ultimate 3000 LC system connected to a ThermoScientific Orbitrap Q-Exactive Plus mass spectrometer in high flow using 2.1 x 50 mm 1.8 mm particle HSS T3 Acquity column and nanoflow using the built-in C18 column.

2.7.1.8 High Flow Rate Analysis of ELA Peptides

Performing the LC-MS analysis of ELA peptides has proved extremely challenging. Their physicochemical properties (highly charged with multiple arginine and lysine residues) result in high interaction with the solid phase material, creating extremely broad peaks using the usual peptide LC-MS reagents and consumables. The ELA-32 and ELA-21 peptides show particularly poor chromatographic separation characteristics using formic acid as a mobile phase modifier and require the addition of TFA to obtain acceptable peaks. The initial metabolite identification was performed using a Thermo Fisher (Thermo Scientific, San Jose, CA, USA) Ultimate 3000 LC system coupled to a Thermo Fisher Q-Exactive plus Orbitrap using the HESI-plus source interface. A Waters Acquity HSS T3 C18 2.1x50 mm column was used for separation of the peptides and was held at a temperature of 60 ºC. Mobile phases used for separation were A: 0.1% Formic acid, 0.01% TFA in water, and B: 0.1% Formic acid, 0.01% TFA in ACN, where initial conditions were 2% B at a flow rate of 300 µl per minute. Peptides were eluted over a 16-minute gradient where the %B was raised to 45% followed by a 2-minute column clean at 90 %B before returning to initial conditions to total a 20-minute run. Full scan data were collected from 400 to 1600 m/z with a resolution of 70,000. A total of 10 µl of the extract was injected for each analysis.
2.7.1.9 **Nanoflow LC-MS/MS Analysis of ELA Peptides**

The extracted peptides were analysed on the same LC-MS system but in a nanoflow mode, with the same mobile phases containing 0.01% TFA. Extracts (30 µl) were loaded onto a 0.3 × 5 mm peptide trap column (Thermo Scientific) at a flow rate of 30 µl/min using 2% B, and washed for 15 min before switching in line with a 0.075 × 250 mm nano easy column (Thermo Scientific) flowing at 300 nl/min. Both nano and trap column temperatures were set at 45°C. Initial conditions were 2% B and held for 15 mins. A ramp to 50% B was performed over 90 mins, and the column then washed with 90% B for 20 mins before returning to starting conditions for a further 20 mins, totalling an entire run time of 130 mins. Positive nanoelectrospray analysis was performed using a spray voltage of 1.8 kV, the tune settings for the mass spectrometer used an S-lens setting of 70 V to target peptides of higher m/z values. A full scan range of 400–1600 m/z was performed at a resolution of 75,000 before the top 10 ions of each spectrum were selected for MS/MS analysis. Existing ions selected for fragmentation were added to an exclusion list for 30 s.

**2.7.2 Apelin Analysis by Mass Spectrometry**

**2.7.2.1 Study Protocol**

This study was registered on Clinicaltrials.gov (NCT03449251) and carried out with ethical approval from the Yorkshire & The Humber – Sheffield Research Ethics Committee (REC reference 18/YH/0010). Six healthy volunteers (3 male and 3 female, mean age 43.8±6.9, with body mass index within the normal range of 23.0±1.0) were recruited for infusion. Volunteers were fasted and were lying supine with their heads supported in a quiet, temperature-controlled (23-25 °C) room for the duration of the study. Following a period of acclimatisation, the first sample of venous blood was obtained from the arm contralateral to the arm used for infusion of apelin. Vials containing [Pyr$^1$]apelin-13 were allowed to warm to room temperature and diluted with physiological saline to produce stock solutions, that were then filtered using a 0.2 µm Portex flat filter (Portex, UK) before undergoing serial dilutions with 0.9% sterile saline.
There was no loss of apelin following this filtration procedure. Volunteers were infused with a concentration of 135 nmol/min of [Pyr\(^1\)]apelin-13, at a rate of 1 ml/min for 120 minutes, using a syringe pump, equipped with a 50 ml syringe and 16 gauge catheter. The second venous sample was obtained immediately after the end of the infusion. Blood samples were collected into 2.6 ml EDTA tubes, immediately put on wet ice and centrifuged (4,000 rpm, 7 mins, 4°C), and stored at -70°C, before analysis. A previous study had used a concentration of up to 100 nmol/min for systemic infusion, where they obtained a therapeutic response in patients with pulmonary arterial hypertension and the highest dose was well tolerated (Brash et al., 2018). The dose chosen of 135 nmol/min of [Pyr\(^1\)]apelin-13, was slightly higher in order to identify possible metabolites. Additional control samples were obtained from 6 donors (3 male and 3 female) within a similar age group who did not receive the apelin infusion for comparison.

### 2.7.2.2 Optimisation of Extraction Methods for Plasma [Pyr\(^1\)]apelin-12 and [Pyr\(^1\)]apelin-13

Four different conditions were used to determine the best method for extracting apelin peptides from human plasma. All sample preparation steps were done on ice to reduce \textit{in vitro} peptide degradation. Human plasma was spiked with 100 ng/ml [Pyr\(^1\)]apelin-13 and transferred (50 µl) to Eppendorf tubes before proteins were precipitated using 300 µl of 75%, 80%, 85% or 90% ACN in water (v/v) (condition 1) or the same ACN solution with 0.1% FA (v/v) (condition 2). In condition 3, 25 µl of guanidine hydrochloride (GuHCl) (6 M) was added to 50 µl plasma and mixed thoroughly before plasma proteins were precipitated with 300µl 80% ACN in water with or without 0.1% FA (v/v). In condition 4, 450 µl water or 50 mM ammonium bicarbonate (pH 8) or 1% FA (v/v) was added to 50 µl plasma and transferred onto Oasis HLB Prime µ-Elution 96-well plates for solid-phase extraction (SPE). The samples from conditions 1-3 were vortexed and centrifuged (12,000 xg, 5 mins, 4°C), and the supernatant transferred to a protein LoBind plate. Samples in condition 3 separated into two liquid layers, both of which were collected. The supernatant was evaporated under a stream of oxygen-free
nitrogen heated to 40 ⁰C using a Biotage SPE dry (Upsala, Sweden) evaporation
system. Dried samples from conditions 2-3 were reconstituted in 200 μl 0.1% FA (v/v)
and loaded unto an Oasis HLB Prime µ-elution 96-well plate (Waters, Wilmslow, UK)
together with samples from condition 4 and slowly extracted on a positive pressure
manifold (Waters). The columns were washed with 200 µl of 5% methanol in water
with 1% acetic acid (v/v) and eluted from the cartridge using 2x 50 µl of 60% methanol
in water with 10% acetic acid. The eluate was evaporated to dryness and reconstituted
in 150 µl 0.1% FA in water, and a volume of 15 µl was injected onto the LC-MS/MS
system.
2.7.2.3

[Pyr1]apelin-13 LC-MS/MS and SRM-based Detection Method
Development on TQX-MS

An LC-MS/MS method was developed for [Pyr1]apelin-13, and its stable isotope
labelled [Pyr1]apelin-13 analogue. LC-MS/MS instrumentation used for the
quantitation of [Pyr1]apelin-13 included an H-Class Acquity (Waters) attached to a TQXS triple quadrupole mass spectrometer (Waters). Peptides were separated using a
2.1 x 50 mm 1.8 mm particle HSS T3 Acquity column held at 60 ⁰C and flowing at 350
µl/minute. Gradient starting condition were 95% A (0.1% FA in water v/v) and 5% B
(0.1% FA in ACN). Starting conditions were held for 0.2 mins before raising to 25% B
over 4 mins. The column was flushed with 90% B for 0.8 mins before returning to
starting conditions. The total time of each analysis was 7 mins, with the first 1.2 mins
and last 2.8 mins diverted to waste. The source parameters used included a positive
electrospray voltage of 3.0 kV, the gas flow of 1000 L/hour, desolvation temperature
of 600 ⁰C and a cone voltage of 40 V.
A full scan analysis of the peptide showed that the [M+4H]4+ charge state was the
predominant ion in the spectrum, and therefore this was selected for fragmentation. A
product ion spectrum was collected over a range of 100 to 1600 m/z, and two ions
were chosen for SRM optimisation (m/z 424.6 and 408.55). The 424.6 ion
corresponded to the b11 fragment and the 408.55 ion was derived from the loss of a
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methyl-sulphide group from the methionine on the b11 ion, as previously described (Mesmin et al., 2010). Optimal conditions for the two SRM transitions for [Pyr\(^1\)]apelin-13 were 384.2/408.55, 384.2/424.6 with collision energy values of 14 and 12 eV respectively. The internal standard used the same collision energy but targeted transitions of 387.45/412.88 and 387.45/428.26. Peptide peak areas were integrated using the TargetLynx program associated with Masslynx V 4.2 (Waters), and peak areas ratios were generated against the corresponding stable isotope-labelled internal standard peptide peak.

2.7.2.4 Extraction of [Pyr\(^1\)]apelin-13 from Human Plasma

Plasma samples were thawed on ice and 50 µl transferred into protein LoBind Eppendorf tubes containing 25 µl GuHCl. A 300 µl aliquot of 80% ACN in water (containing 25 ng/ml internal standard) was added to all plasma samples and vortexed before centrifuging (12000 xg, 5 mins, 4 °C) to precipitate plasma proteins. The supernatant was transferred to a 1 ml protein LoBind 96-well plate and evaporated. Samples were reconstituted in 500 µl 0.1% FA (v/v) and loaded unto an Oasis HLB Prime µ-elution 96-well plate (Waters, Wilmslow, UK) and slowly extracted on a positive pressure manifold (Waters). The columns were washed with 200 µl of 5% methanol in water with 1% acetic acid (v/v) and eluted from the cartridge using 2x 50 µl of 60% methanol in water with 10% acetic acid (v/v). The eluate was evaporated to dryness and reconstituted in 150 µl 0.1% FA (v/v) in water, and 10 µl was injected onto the LC-MS/MS system.

2.7.2.5 Precision and Accuracy of the Extraction Method

Blank plasma was pre-incubated at 37 °C for at least 2 hrs, to degrade endogenous [Pyr\(^1\)]apelin-13 and used to generate an eight-point calibration line of custom synthesised [Pyr\(^1\)]apelin-13 over a range of 0.025-50 ng/ml or 1-1000 ng/ml. A 50 µl aliquot of each calibration standard (0.025, 0.05, 0.1, 0.5, 1, 5, 10 & 50 or 1, 2, 5, 10, 50, 100, 900, and 1000 ng/ml) was extracted using the SPE method described above.
Four levels of quality control (QC) were also generated (1, 3, 100 and 800 ng/ml) and extracted six times in order to assess the precision and accuracy of the method. The order in which QCs and calibrators were analysed, was randomised to remove positional bias. Calibration line followed a linear fit, and $1/x^2$ weighting was applied. Recovery of [Pyr$^1$]apelin-13 from plasma was assessed by analysing spiked solution before and after extraction at a concentration of 100 ng/ml. Plasma samples from six healthy control individuals were also extracted to assess the selectivity of the LC-MS/MS method. The percentage relative error (%RE) was calculated by subtracting the expected concentration from the measured concentration divided by expected concentration and multiplying the result by 100%. Percentage coefficient of variation (imprecision of the assay) was determined by dividing the standard deviation by the mean and multiplying 100%.

2.7.2.6 Peptide Identification Using Orbitrap Mass Spectrometer

Samples were reanalysed on a high-resolution mass spectrometer to identify potential metabolites from the administered [Pyr$^1$]apelin-13 peptide. A full scan analysis was performed using a ThermoScientific Ultimate 3000 LC system connected to a ThermoScientific Orbitrap Q-Exactive Plus mass spectrometer. Solvents used for the separation were A: 0.1% FA in water (v/v) and B: 0.1% FA in ACN (v/v). A volume of 30 μl of the extract was injected onto an HSS T3 UPLC™ column (2.1 × 50 mm; Waters, Elstree, UK) held at 60 °C and with a flow rate of 300 μl/min. A starting condition of 1% B was used to capture the more hydrophilic peptide metabolites, and these were eluted using a linear gradient up to 30% B over 16 mins. The column was washed for 2 mins at 90% B and returned to starting conditions for 2 mins, totalling a run time of 20 mins. Mass spectrometry was performed using positive electrospray mode with a needle voltage of 3 kV, gas settings of 55 and 10 for sheath gas and aux gas flow rates. The temperature of the gas was set at 350°C, and the transfer capillary at 350 °C and an s-lens value of 70 V. Full scan data were acquired over an m/z range of 250–1000, using a resolution of 70,000 and a maximum fill time of 100 ms. Acquired LC-MS data were interrogated for potential [Pyr$^1$]apelin-13 metabolites by searching
for all potential cleavage products from the parent peptide in the RAW data files using PEAKS software, for database searching and Qualbrowser software package (Thermofisher), for manual searching. The potential [Pyr¹]apelin-13 metabolites that were manually identified were subsequently characterised, where 30 μl of the sample was reinjected using a targeted MS/MS analysis. The potential [Pyr¹]apelin-13_{1-6} and [Pyr¹]apelin-13_{1-10} peptides were targeted using precursor ion m/z values of 370.214 (collision energy of 30) and 290.417 (collision energy of 22) respectively. The MS/MS analysis involved the same LC separation, but MS/MS data were acquired at 17,500 resolution with an AGC of 1e6 ions, lowest m/z value of 100 and max fill time of 200 ms.

2.7.3 Peptide Identification using PEAKS Software

LC-MS data were searched using the PEAKS X software (BSI, Waterloo, Canada) against the human Uniprot database (downloaded 18-11-2018) as well as a custom database containing only the apelin or ELA peptide using a no-enzyme setting. This software de novo sequence mass spectra before running the resulting peptide sequence against protein sequences in the UniProt protein database (Ma et al., 2003; Zhang et al., 2012). Where extracts had been reduced and alkylated, a fixed carbamidomethylation modification was applied to cysteine residues. Variable changes included N-terminal acetylation, N-terminal pyroglutamate, C-terminal amidation and methionine oxidation. After initial analyses, it was discovered that in some cases the ELA peptide became over alkylated, where lysine residues acquired additional alkylations (+57.02 Da), and therefore this was added as another variable modification. An FDR setting of 1% was used against a decoy database, and the precursor and product ion tolerances were set as 10 ppm and 0.05 m/z respectively.
2.8 Extraction of Apelin and ELA Peptides from CKD Patients Plasma

In order to reduce the interference of proteins, plasma samples were solid-phase extracted as previously described (Kay et al., 2017; Nyimanu et al., 2019b). Briefly, plasma samples were thawed on ice and 500 µl transferred to a pre-chilled 96-well Protein LoBind plate containing 125 µl of 2 M HCl and vortex-mixed. Plates were then centrifuged (4,000 rpm, 10 mins, 4 °C) and the supernatant loaded onto a 96-well Oasis Primed HLB μ-Elution plate. Samples were slowly extracted on a Waters Positive Pressure 96 Processor (Wilmslow, UK) and washed once with 200 µl of water containing 5% methanol (v/v) and 1% acetic acid (v/v). The resulting samples were eluted into a clean 96-well Protein LoBind plate with 3x 50 µl of 60% methanol and 10% acetic acid in water (%v/v), and centrifuged (1,200 rpm, 2 mins, 4 °C). The eluate was evaporated using Waters SPE Dry 96 System (Wilmslow, UK) under nitrogen and stored at -20 °C until required. The extracts were assayed using the ELA and apelin assay according to manufacturer protocol.

2.9 Enzyme-based Immunosorbent Assay (ELISA)

ELISA was performed to measure levels of ELA in human plasma and tissue homogenates and to measure apelin levels in human plasma. These were done using the commercially available ELISA assay (EK-057-23 and EK-007-19, Phoenix Pharmaceuticals, Burlingame, USA). The apelin ELISA cross-reacts with [Pyr\textsuperscript{1}]apelin-13 and apelin-12, and has an intra-assay and inter-assay variability of <10% and <15% respectively and a sensitivity of 0.07 ng/ml. Also, the ELA ELISA cross-reacts with all the known isoforms of ELA including [pGlu\textsuperscript{1}]ELA-32, ELA-21 and ELA-11 and has an inter- and intra-assay variability of <15% and <10% respectively; minimum detectable concentration of 0.19 ng/ml. The ELISA assay performed according to the manufacturer’s instructions. Briefly, samples either diluted 1:3 in assay buffer or assayed directly were added to wells previously coated with a secondary antibody in duplicates. The primary antibody and biotinylated peptide were subsequently added
and incubated with rotation (300-400 rpm) for 2 hrs. After washing and blot drying, streptavidin-conjugated horseradish peroxidase (SA-HRP) was added and incubated for 1 hr with rotation before the substrate solution tetramethylbenzidine (TMB) was added after another round of washes and incubated for a further 1 hr. The reaction was quenched by adding 2 N HCl into each well, and absorbance at 450 nm was measured using a Synergy HT microplate reader (Biotek, Vermont, USA). The absorbance was proportional to the amount of biotinylated peptide–peroxidase complex and therefore, inversely proportional to the concentration of peptides in the samples. Unknown concentrations were determined by interpolation to a standard curve of known concentrations fitted to a 4-parameter logistic concentration-response curve in GraphPad Prism 6.

### 2.10 Left Ventricular Catheterisation for Haemodynamics Measurement in Anaesthetised Rats

To assess the *in vivo* actions of apelin peptides and its analogues, left ventricular catheterisation experiments were conducted on normotensive male Sprague Dawley rats. Following seven days of acclimatisation, the rats were anaesthetised with gaseous isoflurane (5% for induction, 3% for initial maintenance before surgery and 1.5-2% for maintenance during surgery; 1.5L/min oxygen). Rats were placed on a heat mat, and body temperature monitored using a rectal thermometer and maintained between 36-37 °C throughout the study. The depth of anaesthesia and cessation of pain reflexes were assessed by two methods, using toe-pinching and eye reflexes (blinking) test before surgery and monitored throughout the surgery by the rate of breathing and, following catheterisation, heart rate. The pressure-volume catheter (SPR-869, Millar Inc, Oxford, UK) was connected to a data PowerLab 16/35 system with the LabChart 8 software (ADInstruments, Oxford, UK), calibrated with the MPVS Ultra PV Unit (ADInstruments, Oxford, UK) and left ventricular catheterisation performed as previously described (Pacher et al., 2008; Yang et al., 2017b, 2019). In brief, with the rat lying in the supine position, the right external jugular vein was
exposed and cannulated with a polyethylene tubing (Smiths Medical) containing heparinised 0.9% saline solution. This was used for bolus administration of compounds into the animal. The right carotid artery was then isolated for insertion of a pressure-volume catheter into the left ventricle. The position of the catheter was determined by the shape of the pressure-volume trace, heart rate and pressure recordings. Once in the right position, it was allowed approximately 10-15 mins to stabilise before injection of compounds. A dose of saline (0.9%, 0.5 ml) or relevant compound at an increasing dose (0.5 ml each, 2 doses) was administered and responses recovered for 10-15 mins or until it has returned to baseline. Following injection, the cannula was washed with 0.1 ml saline (0.9%). At baseline, a final dose of [Pyr1]apelin-13 (50 nmol, 0.5 ml) was administered as described above. The animal was sacrificed by exsanguination followed by cranial dislocation after taking recordings. The data were analysed as previously described (Read et al., 2016), and the peak effects of compounds on the left ventricular systolic pressure, cardiac output, heart rate, contractility (dp/dt\text{max}) and stroke volume were expressed as change from baseline.

2.11 Statistical Analysis

Data were expressed as pD$_2$±SEM for cell-based assays and repeated at least three times in duplicate. Binding data were described as pK$_i$±SEM. Differences between mean pD$_2$ of each analogue compared to native apelin-36 were analysed using a One-Way ANOVA with Dunnet’s or Tukey’s multiple comparisons test in GraphPad Prism 6. All other data were expressed as mean±SEM and statistical analysis performed using either Student’s t-test or ANOVA, or their nonparametric equivalent as required in Graphpad Prism. A p value < 0.05 was considered statistically significant.
Chapter 3 Characterisation of the Apelin System in Health and Disease

3.1 Introduction

Apelin and apelin receptor are widely expressed in various tissues including kidney, heart, lungs and brain. In the cardiovascular system, apelin is a vasodilator and positive inotrope (Maguire et al., 2009; Read et al., 2019a), and control renal haemodynamics in the kidney (Hus-Citharel et al., 2008). Apelin also promote diuresis (Hus-Citharel et al., 2014) and increase insulin sensitivity (Bertrand et al., 2015; Nyimanu et al., 2019a). However, the unexpected phenotypic differences between apelin and apelin receptor mutants suggested that another ligand might activate the apelin receptor. Consequently, ELA was discovered in zebrafish as the second endogenous ligand of the apelin receptor, essential for the proper development of the heart (Chng et al., 2013; Pauli et al., 2014). ELA expression was proposed to be exclusive to the prostate and kidney in adult mammals (Chng et al., 2013; Deng et al., 2015; Wang et al., 2015). Although its role in the prostate is unknown, it was later suggested that ELA promote diuresis in rats (Deng et al., 2015). Moreover, recent studies indicate that ELA was not only required for mammalian cardiovascular development (Freyer et al., 2017; Ho et al., 2017) but also placentation and that ELA deficient mice developed preeclampsia, which was reversed by exogenous ELA (Ho et al., 2017). Notably, both studies suggested that ELA deficiency in mice was embryonic lethal with a subset of pups surviving to adulthood.

The apelin receptor-signalling pathway was proposed to regulate the renin-angiotensin system negatively. Apelin receptor directly interacts with the angiotensin receptor by heterodimerisation to inhibit angiotensin II-mediated cardiac hypertrophy and vasoconstriction (Siddiquee et al., 2013). In heart failure, apelin increased the expression of angiotensin-converting enzyme 2, which converts angiotensin-II to the cardioprotective angiotensin 1-7 (Sato et al., 2013). Recently, ELA was shown to not
only have cardioprotective effects in pressure overload induced-heart failure but also directly decrease the expression of angiotensin-converting enzyme (ACE) at mRNA and protein level via inhibitory actions on ACE transcription factor FoxM1 (Sato et al., 2017). Additionally, ELA infusion was beneficial against acute kidney disease (Chen et al., 2017). These studies suggest that ELA may have a similar effect as ACE inhibitors currently used in the clinical management of cardiovascular and renal diseases and raised the possibility that modulation of ELA-apelin receptor signalling pathway may be a potential therapeutic strategy in kidney diseases.

Acute kidney injury which often progresses into CKD is commonly caused by ischaemia/reperfusion injury in human kidney and is responsible for over 2 million deaths worldwide (Chen et al., 2015b; Czopek et al., 2016). Clinically, CKD is diagnosed as kidney damage ≥3 months confirmed by biopsy or makers of kidney damage (e.g. proteinuria), or decreased glomerular filtration rate (GFR, <60 ml/min/1.73 m²) for ≥3 months and classified into five stages (Sarnak et al., 2003) (Table 3.1). Importantly, there is no treatment for chronic kidney disease, and identification of a novel therapeutic target may have a significant clinical benefit to patients.

### Table 3.1. Classification of chronic kidney disease

<table>
<thead>
<tr>
<th>Stages</th>
<th>Description</th>
<th>GFR, ml/min/1.73 m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Kidney damage with normal GFR</td>
<td>≥90</td>
</tr>
<tr>
<td>2</td>
<td>Kidney damage with mildly decreased GFR</td>
<td>60-89</td>
</tr>
<tr>
<td>3</td>
<td>Kidney damage with moderately decreased GFR</td>
<td>30-59</td>
</tr>
<tr>
<td>4</td>
<td>Severely decreased GFR</td>
<td>15-29</td>
</tr>
<tr>
<td>5</td>
<td>Kidney failure</td>
<td>&lt;15 or dialysis</td>
</tr>
</tbody>
</table>
GFR, glomerular filtration rate. The normal GFR is \(~125 \text{ ml/min}^{1}/1.73 \text{ m}^{2}\) (Samak et al., 2003)

3.1.1 Hypotheses and Aims

Studies in animal models have shown that modulation of the apelin receptor may protect the kidney from kidney injury and potentially delay the progression to chronic kidney disease. Thus, targeting the apelin receptor signalling pathway by apelin or ELA may be an important therapeutic strategy for clinical management of renal diseases. However, the role of the apelin system (both ligands and receptor) in renal physiology is mainly unknown. In addition, the expression of the apelin system, especially at the protein level within the kidney is not well understood, and this could be used to infer physiological functions of the apelin system in the kidney.

I hypothesised that although initial studies suggested ELA was expressed mainly in the kidney, it was present in other tissues and that both ELA and apelin may have compensatory effects.

The main aims of this study were:

1. To find potential evidence for expression of ELA in other tissues and compare this to levels of apelin and apelin receptor.
2. Investigate any potential changes in the levels of Ace2 and Ace in Apela deficient mouse kidney and heart.
3. Further, characterise the localisation of ELA in human kidney compared to apelin and apelin receptor.
4. Evaluate whether there were any changes in apelin pathway in chronic kidney disease using disease models and patient samples.
3.2 Materials and Methods

3.2.1 Materials

All materials used in this chapter were listed in section 2.1. Also, Dr Dhaun Neeraj, of the University of Edinburgh, provided human plasma samples obtained from control and chronic kidney disease patients. Mouse distal convoluted tubule (mDCT 209) cells were a gift from Dr Meena Murphy, at the University of Cambridge.

3.2.2 Gene Expression Analysis

Total RNA were extracted from human kidney medulla (n=6) and cortex (n=6), whole kidney (n=5), brain (n=11), lungs (n=9), left ventricle (n=9), right ventricle (n=9), and WT (n=6), Apela heterozygous (Apela<sup>+</sup> n=8) and Apela KO (Apela<sup>-</sup> n=8) mouse brain, kidney, heart, spleen and liver as described in section 2.3. Following cDNA synthesis (section 2.3.2), real-time quantitative PCR experiments (section 2.3.3) were performed using specific primer probes for human and mouse Apln, Apela and Aplnr gene as well as mouse Ace and Ace2 genes. The primer sequences and IDs are listed in section 2.3.3 table 2.1.

3.2.3 Dual Immunofluorescence Staining of Human Kidney

Human kidney (n=4-5) sections were stained as described in section 2.4.1 and imaged. The expression of apelin pathway protein along different regions of the renal nephron was determined using specific markers (Table 3.2).
Table 3.2. Cell-specific markers used to determine the cellular localisation of apelin, ELA and apelin receptor in human kidney.

<table>
<thead>
<tr>
<th>Protein (human)</th>
<th>Region marked</th>
</tr>
</thead>
<tbody>
<tr>
<td>Von-willebrand factor (vWF)</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>Aquaporin 1 (AQP1)</td>
<td>Proximal convoluted tubule and thin descending limb of the loop of Henle</td>
</tr>
<tr>
<td>Aquaporin 4 (AQP4)</td>
<td>Collecting duct</td>
</tr>
<tr>
<td>Sodium potassium chloride cotransporter (NKCC2)</td>
<td>Thick ascending limb of the loop of Henle (TAL)</td>
</tr>
<tr>
<td>Transient receptor potential cation channel subfamily m member 6 (TRPM6)</td>
<td>Distal convoluted tubule</td>
</tr>
<tr>
<td>Sodium-glucose cotransporter 2 (SGLT2)</td>
<td>Segment 1 of the proximal convoluted tubule</td>
</tr>
</tbody>
</table>

3.2.4 Immunofluorescence Staining of Mouse Kidney Cells

Mouse DCT-209 cells (P43) were seeded at 4 x 10^5 cells/well in 6-well plates containing coverslips previously coated with Poly-L-lysine (1 ml; 5 mins). All experiments were performed at room temperature unless specified. Cells were seeded overnight and fixed in ice-cold acetone: methanol (1:1 ratio) for 5 mins at room temperature and unspecific binding blocked with 5% goat serum in PBS (2 ml/well; 2 hrs). Primary antibodies against human apelin, ELA and apelin receptor were prepared in a buffer (PBS containing 0.01% Tween and 3% goat serum) and added to the cells (1 ml/well) before incubating overnight at 4°C. Primary antibodies used include rabbit anti-human apelin receptor (1:100), apelin (1:50) and ELA (1:300). Following washing (3x 5 mins), goat anti-rabbit Alexa Fluor-488 (1:200) and Hoechst (1:100) prepared in
buffer were added (1 ml/well) and incubated for 1 hr. Slides were washed (3x 5 mins) and mounted with ProLong Gold Antifade reagent before imaging on Zeiss 510 Meta confocal laser scanning microscope (Heidelberg, Germany) at x40 magnification. Images were processed using Fiji (ImageJ) for background subtraction using rolling ball method and channels merged.

### 3.2.5 Receptor Pharmacology Studies

#### 3.2.5.1 Receptor Autoradiography

Human kidney sections (n=6) and *Apela* knockout and heterozygous mice kidney were incubated with 0.5 nM \[^{125}\text{I}]\text{apelin-13}\) for 90 mins in binding buffer (section 2.6.3). Tissue sections were incubated for 1-2 weeks in the dark and films processed as described in section 2.6.3 and imaged.

#### 3.2.5.2 Saturation Experiments

Human kidney (n=3) was homogenised, as described in section 2.5.1. \[^{125}\text{I}]\text{apelin-13}\) (2nM - 3.9pM) was diluted in a two-fold serial dilution and split in two before \([\text{Pyr}^1]\text{apelin-13}\) (2 µM) was added to one half to determine nonspecific binding and the other half used as total binding. Increasing concentrations of nonspecific and total binding samples were incubated with protein homogenates as described in section 2.6.1 and bound radioactivity counted.

### 3.2.6 Chronic Kidney Diseases

#### 3.2.6.1 Mouse Model

The mouse model ischaemia/reperfusion injury-induced chronic kidney disease was provided by Dr Dhaun (University of Edinburgh). Briefly, ischaemia reperfusion injury was induced in male FVB mice (n=8) by midline laparotomy and 50 mins clamping of left renal pedicles as previously described (Clef et al., 2016; Zager et al., 2013), followed by 4 weeks of reperfusion. Tissues recovered after sacrificing animals were used for RT-qPCR analysis.
3.2.6.2 CKD Patient Plasma ELISA

Plasma obtained from 128 patients with CKD (mean age, 46 ± 1; male/female, 80/48) and 27 healthy volunteers (mean age, 47 ± 2; male/female, 15/12) were solid-phase extracted as described in section 2.10. Following extraction, ELISA was performed on these samples to assess any changes in the plasma levels of apelin or ELA.

3.2.7 Statistical Analysis

Data were expressed as mean±SEM. All statistical analysis was performed in Graphpad Prism (v6). For statistical analysis, all data were first tested for normality using Graphpad prism’s recommended D’Agostino-Pearson omnibus K² test. This test accounts for skewness and kurtosis of the data and determines how much individual value differs from that expected in a normal distribution and requires minimum n value of 8. Hence, where the n value was less than 8, the Shapiro-Wilk normality test was used. Where the data were not normally distributed, a non-parametric test was performed. For example, data obtained from the CKD mouse model was not normally distributed, hence the non-parametric equivalent of paired t-test, Wilcoxon matched-pairs signed-rank test was performed. For comparison of two datasets, the student’s t-test or Mann Whitney test was performed while for ≥3 datasets, One-Way ANOVA with Tukey’s multiple comparisons or Kruskal-Wallis test was performed. Correlation analyses were performed using either Pearson’s or Spearman’s correlation depending on whether or not data were normally distributed.

Saturation binding data were analysed, as described in section 2.6.1.
3.3 Results

3.3.1 Expression of Apelin, Apela (ELA) and Apelin Receptor in Mouse Tissues

It is well known that the apelin receptor and apelin are ubiquitously expressed in the body (Chng et al., 2013; Deng et al., 2015) but the expression profile of ELA is mostly unknown. Hence, to further investigate the expression of these ligands and receptor, adult Apela knockout mice were obtained from Dr Hadjantonakis (Sloan Kettering Institute, New York, USA). The mRNA expression level in five essential organs of these mice was investigated and compared to wildtype (WT) or heterozygous (Het) control, as shown below.

3.3.1.1 Mouse Brain

In wildtype mice, both Apela and Apln were expressed in the brain. However, Apln levels were significantly higher (~24 fold) than the Aplnr and over 3-fold higher than Apela (Fig. 3.1A). However, in brain tissue obtained from Apela heterozygous mice, there was also a significant difference in the levels of Apln compared to Apela or Aplnr (Fig. 3.1B). As would be expected, Apela was not detected in the Apela knockout brain, but there was a significant decrease in the expression of Aplnr compared to Apln in these mice (Fig. 3.1C). Additionally, compared to wild type, in Apela heterozygous and knockout brain tissues, there was a trend for reduced Apln levels, but this was not significant (Fig. 3.1D). Also, there was no change in the Aplnr expression when compared to wildtype or heterozygous (Fig. 3.1E).
Figure 3.1. mRNA expression of apelin pathway genes in mouse brain tissues. Expression of the ligands and their receptor in wildtype (A), heterozygous (B) and Apela knockout mice (C). Compared to wildtype, there was no difference in Apln expression in Het or KO brain (D). There was a trend for decreased Apln expression in Het or KO brain (E) compared to WT. WT, Wildtype; Het, heterozygous; KO, knockout.* indicates p<0.05, ** indicates p<0.001, *** indicates p<0.0001; n = 6 WT,
8 Het and KO. Data expressed as relative expression compared to β-actin, used as housekeeping and shown as mean±SEM.
3.3.1.2  Mouse heart

*Apela* mRNA levels were significantly lower in the wildtype and heterozygous heart only when compared to *Aplnr*, but there was no difference compared to *Apln* levels (Fig. 3.2A-C). Although not significant, there was a trend for increased expression of both *Apln* and *Aplnr* in *Apela* knockout mice heart (Fig. 3.2D-E).
Figure 3.2. mRNA expression of apelin pathway genes in mouse heart tissues. Expression of the ligands and their receptor in wildtype (A), heterozygous (B) and Apela knockout mice (C). There was a trend for increased expression of Aplnr (D) and Apln (E) the knockout (KO) heart compared to wildtype (WT) or heterozygous (Het). * indicates $p<0.05$, ** indicates $p<0.001$, *** indicates $p<0.0001$; $n = 6$ WT, 8 Het and
KO. Data expressed as relative expression compared to β-actin, used as housekeeping gene and shown as mean±SEM.
3.3.1.3 Mouse Kidney

Both Apln and Aplnr were present in the mouse wildtype kidney at low levels (~40-fold and ~85-fold lower respectively) when compared to Apela. Apela was expressed at very high levels in the mouse kidney (Fig. 3.3A). In Apela heterozygous kidney, about half of those present in the wild type was detected, and no Apela mRNA was found in kidney samples obtained from knockout animals (Fig. 3.3A-C). Additionally, Apln but not Aplnr expression was significantly reduced in Apela knockout kidney when compared to wildtype (Fig. 3.3D-E).
Figure 3.3. mRNA expression of apelin pathway genes in mouse kidney tissues. Expression of the ligands and their receptor in wildtype (A), heterozygous (B) and Apela knockout mice (C). There was no difference in Aplnr in heterozygous (Het) and knockout (KO) kidney (D). Apln expression was significantly lower in Apela knockout (KO) compared to wildtype (WT) (E). * indicates p<0.05, ** indicates p<0.001, *** indicates p<0.0001; n = 6 WT, 8 Het and KO . Data expressed as relative expression compared to β-actin, used as housekeeping gene and shown as mean±SEM.
3.3.1.4 Mouse spleen and liver

Interestingly, neither Apln nor Apela was expressed in the liver and spleen. However, Aplnr was not only present, but its expression was over two-fold higher in the Apela deficient liver compared to WT or Het (Fig. 3.4A-B). In these tissues, there were no changes in the expression level of the Aplnr in wildtype, heterozygous spleen and liver.

![Figure 3.4](image)

Figure 3.4. mRNA expression of apelin pathway genes in mouse spleen (A) and liver (B) tissues. Apln and Apela expression were not detected in either wildtype (WT), heterozygous (Het) or knockout (KO); n = 6 WT, 8 Het and KO each. Data expressed as relative expression compared to β-actin, used as housekeeping gene and shown as mean±SEM.

3.3.1.5 Mouse Lung

In the wildtype lung, Apln was the highest expressed gene, followed by the Aplnr. The mRNA levels of both Apln and the Aplnr were significantly higher than that of Apela gene in this tissue (Fig. 3.5). Apela knockout and heterozygous mice were not available; hence only the wildtype data are shown.
Figure 3.5. mRNA expression of Apln, Aplnr and Apela in wildtype mouse lung. Data expressed as relative expression compared to β-actin, used as housekeeping gene and shown as mean±SEM; * indicates p<0.05; n=6.

3.3.2 Apela Deletion Alters Ace/Ace2 Ratio in Mice Kidney but not Heart

In the kidney, Ace2 mRNA expression was significantly increased compared to Ace while this trend was reversed in the heart, where a significant increase in mRNA expression of Ace compared to Ace2 was observed (Fig. 3.6A, B). Additionally, although the levels of Ace mRNA were similar in the heart compared to the kidney, Ace2 mRNA expression was significantly upregulated compared to Ace in the kidneys especially in heterozygous and Apela knockout mice (Fig. 3.6C, D). Additionally, significantly high Ace/Ace2 ratio were observed in the Apela knockout kidney but not in the heart (Fig. 3.6E, F), suggesting increased expression of renal Ace in the absence of Apela.
Figure 3.6. mRNA expression of Ace and Ace2 in mouse heart and kidney tissues. Expression of Ace and Ace2 in the heart (A) and kidney (B); C, comparison of Ace2 expression levels in the heart and kidney; D, comparison of Ace expression levels in the heart and kidney; ACE/ACE2 ratio in the heart (E) and kidney (F). Wildtype (WT), heterozygous (Het) and knockout (KO) kidney. * indicates $p<0.05$, ** indicates $p<0.001$, *** indicates $p<0.0001$; $n = 6$ WT, 8 Het and KO each. Data expressed as
relative expression compared to β-actin, used as housekeeping gene and shown as mean±SEM.
3.3.3 Distribution of $[^{125}\text{I}]$Apelin-13 Binding Sites in Knockout and Heterozygous Mouse Kidney

To further determine whether there were any changes in the density of the receptor in the kidney of Apela KO compared to Apela Het mice, autoradiography was performed on kidney sections. As expected, no binding was observed in the presence of unlabelled [Pyr$^1$]apelin-13 used to define nonspecific binding (Fig. 3.7A, C). Dense distribution of $[^{125}\text{I}]$apelin-13 binding sites were observed in the inner medulla of both knockout and heterozygous kidney (Fig. 3.7B, D). The density of the apelin receptor in the KO compared to Het kidney did not appear to differ significantly, suggesting that Apela KO may not have affect receptor density. There was no evidence of $[^{125}\text{I}]$apelin-13 binding in the cortex and outer medulla in these mice.
3.3.4 Translation of Expression Profile to Humans

Having characterised the expression profile of the Apln, Apela and Aplnr gene in mouse tissues, I next wanted to see how these translated into humans. Hence, human tissues were analysed for expression of the apelin pathway genes by RT-qPCR.
3.3.5 Expression of \textit{APLN}, \textit{APELA} and \textit{APLNR} in Human Tissues

\subsection*{3.3.5.1 Lung and Brain}

In human lung, the expression level of \textit{APLN} and \textit{APELA} were very similar. However, \textit{APLNR} expression was five-fold higher than that of \textit{APLN} and over six-fold higher than \textit{APELA} (Fig. 3.8A). Additionally, the levels of \textit{APLN} and \textit{APLNR} were very similar in the brain, but the mRNA expression of \textit{APELA} was significantly lower compared to both \textit{APLN} and \textit{APLNR} (Fig. 3.8B).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.8.png}
\caption{mRNA expression of the \textit{APLN}, \textit{APELA} and \textit{APLNR} in human lung (A) and brain (B). * indicates \textit{p}<0.05, ** indicates \textit{p}<0.001, *** indicates \textit{p}<0.0001; \(n = 9\) (lung); \(n = 11\) (brain). Data expressed as relative expression compared to \(\beta\)-actin, used as housekeeping gene and shown as mean±SEM.}
\end{figure}

\subsection*{3.3.5.2 Left and Right Ventricle of the Heart}

In the human left and right ventricle, \textit{APLN}, and \textit{APLNR} levels were significantly higher than that of \textit{APELA} (Fig. 3.9A, B). \textit{APLN} levels were significantly lower compared to \textit{APLNR} in the right ventricle, but there was no difference in the left ventricle. Additionally, \textit{APELA} levels were significantly higher in the left compared to the right ventricle (Fig. 3.9C), but there was no difference between the expression of either \textit{APLN} or \textit{APLNR} in these anatomical regions (Fig. 3.9D, E).
Figure 3.9. Expression of APLN, APELA and APLNR mRNA in the human heart. Expression in the right (A) and left (B) ventricle; comparison of expression levels of APELA (C), APLN (D) and APLNR (E) in the left and right ventricle. NRV, normal left ventricle (n=9); NLV, normal right ventricle (n=9). * indicates p<0.05, ** indicates...
p<0.001, *** indicates p<0.0001. Data expressed as relative expression compared to β-actin, used as housekeeping gene and shown as mean±SEM.
3.3.5.3 Kidney

To characterise the expression of the apelin pathway genes in human kidney, the kidneys were first separated into cortex and medulla to see whether there was any anatomical difference in expression profile. It was observed that APLN, APELA, and APLNR were expressed at similar levels in both cortex and medulla (Fig. 3.10A-B). Whole kidney sections were also investigated, but still, there was no difference in the mRNA expression level of APELA compared to either APLN or APLNR (Fig. 3.10C).

Figure 3.10. Expression of APLN, APELA and APLNR mRNA in human kidney. A, cortex (n=6); B, medulla (n=6); and C, whole kidney (n=5). Data expressed as relative
expression compared to β-actin, used as housekeeping gene and shown as mean±SEM.

3.3.6 Cellular Localisation of Apelin Pathway Proteins in Human Kidney

Data from the qPCR studies suggested that APELA was most predominantly expressed in the kidney, especially in mice. However, in human tissues, there was no significant difference between the levels of APELA compared to APLN or APLNR, although there was a trend for increased APELA expression. Also, the mRNA expression profile does not provide any information on where in the kidney, these genes are expressed. Furthermore, the localisation of apelin system within the kidney especially at the protein level is poorly understood, and this knowledge is highly needed for prediction of potential renal functions of the apelin system, especially the newly discovered ELA. Hence, the cellular distribution of ELA relative to apelin and apelin receptor along the different segments of the renal nephron was investigated further using specific markers (Fig. 3.11).
Figure 3.11. Schematic of a renal nephron showing the different segments and specific proteins used to determine apelin system expression in each region. EC, endothelial cells; vWF, von Willebrand factor; PCT, proximal convoluted tubule; AQP1, aquaporin 1; SGLT2, sodium-glucose cotransporter 2; LoH, Loop of Henle; TAL, thick descending Loop of Henle; DCT, distal convoluted tubule; TRPM6, transient receptor potential cation channel subfamily M member 6; AQP4, aquaporin 4.

3.3.6.1 The Localisation of Apelin, Apelin Receptor and ELA in Renal Blood Vessels

Dual fluorescence immunolabelling was performed to localised apelin, ELA and apelin receptor in specific cell types of human kidney blood vessels. Apelin (Fig. 3.12E), ELA (Fig. 3.12I) and apelin receptor (Fig. 2.12N) immunoreactivity were present in renal blood vessels and these colocalised with von Willebrand factor (vWF) staining in the endothelium (Fig. 3.12H, M, Q). Negative control slides were primary antibody was omitted but contained secondary antibody, and Hoechst did not show any positive staining in the green and red channel as expected (Fig. 3.12A-D). Additionally, expression of the apelin receptor was observed in the smooth muscle component of the vessel (Fig. 3.12N-Q), but this staining was not detectable with apelin or ELA antibody (Fig. 3.12E-M).
Figure 3.12. Expression of apelin, ELA and apelin receptor protein in renal blood vessels. Apelin, apelin receptor and ELA immunoreactivity colocalised with vWF in the endothelium of human kidney; n=4-5. –ve control, negative control where the primary antibody was omitted, vWF, von Willebrand factor as a marker of endothelial cells.
3.3.6.2 Glomerular Expression of Apelin, ELA and Apelin Receptor in Human Kidney

Punctate staining of apelin (Fig. 3.13A-E), ELA (Fig. 3.13F-J) and apelin receptor (Fig. 3.13K-O) were observed in the glomerulus and blood vessel. Interestingly, the apelin, ELA or apelin receptor immunoreactivity in the glomerulus does not appear to colocalise with von Willebrand factor (vWF), an endothelial marker. However, there was colocalisation in the blood vessel (Fig. 3.13D, I, N).
Figure 3.13. The localisation of apelin, ELA and apelin receptor in renal blood vessel and glomerulus. vWF, von Willebrand factor as a marker of endothelial cells. E, J, O; Single glomerulus (Glom). Scale bar = 50µm; magnification, x40; n=4-5.
3.3.6.3 The Localisation of Apelin Pathway Proteins in Human Kidney

Proximal Convoluted Tubule

In the kidney, aquaporin 1 (AQP1) is expressed in the epithelial cells of the proximal convoluted tubule and thin descending limb of the Loop of Henle (LoH) (Nielsen et al., 2002). Hence, in the renal cortex, a large tubule staining for AQP1 is the proximal tubule. Here, apelin (Fig. 3.14 A-D), ELA (Fig. 3.14F-I) and apelin receptor (Fig. 3.14K-N) staining were observed in these large cortical tubules. This staining colocalised with AQP1 staining in these sections (Fig.3.14D, F, N), identifying the tubules as the proximal convoluted tubule. Furthermore, more apparent glomerular staining of apelin, ELA and apelin receptor was observed (Fig. 3.14E, J, O).

Furthermore, the sodium-glucose transporter, SGLT2, is expressed in segment 1/2 of the proximal tubule and has recently been explored as a therapeutic target for the treatment of diabetes (Gallo et al., 2015). Strong apelin receptor expression was observed in cortical tubules, and this colocalised with SGLT2 staining (Fig. 3.14Q-T).

In addition, strong apelin receptor expression was observed in juxtaglomerular structures (Fig. 3.14O) and another unidentified structure (Fig. 3.14U), which might be part of the distal tubule.
Figure 3.14. The localisation of apelin, ELA and apelin receptor in the proximal convoluted tubule of human kidney; Arrow indicates juxtaglomerular apparatus; AQP1, aquaporin 1, Glom, glomerulus; SGLT2, sodium-glucose cotransporter 2, APLNR, apelin receptor; n=4-5.
3.3.6.4 The Localisation of Apelin Pathway Proteins in Human Kidney Loop of Henle

Descending limb of the loop of Henle is a thin tubule present in the outer medulla and some parts of the inner medulla, which as earlier mentioned express the water channel aquaporin 1 (AQP1). Here, it was observed that apelin (Fig. 3.15A), ELA (Fig. 3.15E) and apelin receptor (Fig. 3.15I) appeared to be expressed in these tubules and colocalised with AQP1 in the medulla (Fig. 3.15D, H, L).

Figure 3.15. The localisation of apelin, ELA and apelin receptor in the descending limb of the loop of Henle. AQP1, aquaporin 1 used as a marker; APLNR, apelin receptor; n=4-5.
3.3.6.5 Apical and Basolateral Localisation of Apelin, ELA and Apelin Receptor in the Proximal Tubule and Descending Limb of the Loop of Henle

To ascertain whether apelin, ELA and apelin receptor was localised in the apical and basolateral membrane of the proximal renal tubule and descending limb of Loop of Henle, paraffin-embedded (waxed) cortical sections were obtained from Professor Fiona Karet, University of Cambridge. Using these sections, the expression of apelin, ELA and apelin receptor in the proximal tubule and descending limb of the Loop of Henle were confirmed. It was also observed that apelin appeared to be expressed mainly in the luminal or apical membrane (Fig. 3.16A-D), while ELA and apelin receptor was expressed in both the apical and basolateral membrane (Fig. 3.16E-L).
Figure 3.16. Apical and basolateral membrane expression of apelin, ELA and apelin receptor in the proximal tubule and descending limb of the loop of Henle. APLNR, apelin receptor, AQP1, aquaporin 1 used as a marker of the proximal tubule and descending limb of the loop of Henle; n=4-5.

3.3.6.6 The Localisation of Apelin Pathway Proteins to the Thick Ascending Limb of the Loop of Henle (TAL)

The sodium-potassium chloride (Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\)) cotransporter (NKCC2) is expressed in the thick ascending limb of the loop of Henle (TAL) and often used as a specific marker of this region of the nephron (Castrop and Schießl, 2014). In this study, an intense staining of apelin (Fig. 3.17A), ELA (Fig. 3.17E) and apelin receptor (Fig. 3.17I) were
observed which colocalised with NKCC2 staining (Fig. 3.17D, H, L), suggesting that apelin, ELA and apelin receptor are localised in this region of the nephron.

Figure 3.17. The localisation of apelin, ELA and apelin receptor in the thick ascending limb of the loop of Henle. APLNR, apelin receptor; NKCC2, Na⁺-K⁺-2Cl⁻ cotransporter; n=4-5.
3.3.6.7 The Localisation of Apelin Pathway Proteins in Collecting Duct

Intense staining for apelin (Fig. 3.18A), ELA (Fig. 3.18E) and apelin receptor (Fig. 3.18I) were observed in the renal medulla. This colocalised with aquaporin 4 staining, used as a marker of renal collecting duct principal cells (Fig. 3.18D, H, L).

Figure 3.18. The localisation of apelin, ELA and apelin receptor to the renal collecting duct. AQP4, aquaporin 4 used as a marker of collecting duct principal cells; APLNR, apelin receptor; n=4-5.
3.3.6.8 The Localisation of the Apelin Receptor in Distal Convoluted Tubule and Juxtaglomerular Apparatus

An intense apelin receptor staining was observed in the cortex (Fig. 3.19A, E, I). This colocalised with staining for the magnesium channel, transient receptor potential cation channel subfamily M member 6 (TRPM6), a specific marker of the distal convoluted tubule. Also, a strong expression of apelin receptor was observed in the juxtaglomerular cells (Fig. 3.19E-H; I-L).
Figure 3.19. Expression of the apelin receptor in the distal tubule and juxtaglomerular apparatus. Arrow indicate cells of the juxtaglomerular apparatus; TRPM6, Transient receptor potential cation channel subfamily M member 6; AQP1, aquaporin 1; APLNR, apelin receptor; n=4-5.

Staining of the distal tubule for apelin and ELA peptides was not very convincing (data not are shown). Hence, to further determine the expression of these ligands in the distal convoluted tubule, mouse distal convoluted tubule cells were stained for apelin, ELA and apelin receptor. Punctate staining of the apelin (Fig. 3.20A-C), ELA (Fig. 3.20D-F) and apelin receptor (Fig. 3.19G-I) were observed in these cells. Negative controls were the primary antibody was omitted, showed that the observed apelin, ELA and apelin receptor staining were specific (Fig. 3.20J-L).
Figure 3.20. Expression of apelin, ELA and apelin receptor in mouse distal convoluted tubule cells. APLNR, apelin receptor; -ve control, negative control where the primary antibody was omitted. Arrows indicate punctate staining; n=2.
**3.3.7 Distribution of $[^{125}\text{I}]$Apelin-13 Binding Sites in Human Kidney**

Autoradiography was performed to determine the distribution of apelin receptor binding sites in whole kidney sections. Dense distribution of apelin receptor binding sites was observed predominantly in the cortex (Fig. 3.21A). A light patch of binding sites was present in the medulla and renal blood vessels (Fig. 3.21A). In the presence of [Pyr$^1$]apelin-13, binding was abolished entirely, suggesting that observed binding was specific (Fig. 3.21B). $[^{125}\text{I}]$apelin-13 was used instead of radiolabelled ELA in these receptor-binding experiments because there is currently no available radiolabelled ELA.

![A](image1.png) ![B](image2.png)

*Figure 3.21. Representative distribution of apelin receptor in the human kidney by $[^{125}\text{I}]$apelin-13 binding sites. A, total binding; B, nonspecific binding. Each black dot represents the apelin receptor binding site. Arrow indicates binding to a blood vessel; n=6.*

Next, the saturation binding experiment was performed to determine the density of the apelin receptor in human kidney. [Pyr$^1$]apelin-13 bound the apelin receptor in the human kidney with a single Hill slope (Table 3.3). In addition, [Pyr$^1$]apelin-13 had a
subnanomolar affinity (K\textsubscript{D}) for its receptor in the human kidney, and the density of the receptor was 15.41±2.29 Fmol/mg in this tissue (Table 3.3).

**Table 3.3. Apelin receptor density in the kidney.**

<table>
<thead>
<tr>
<th>K\textsubscript{D} (nM)</th>
<th>B\textsubscript{Max} (fmol/mg)</th>
<th>Hill Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.33</td>
<td>19.99</td>
<td>0.989</td>
</tr>
<tr>
<td>0.205</td>
<td>12.93</td>
<td>1.000</td>
</tr>
<tr>
<td>0.20</td>
<td>13.31</td>
<td>1.142</td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>0.34±0.13</td>
<td>15.41±2.29</td>
</tr>
</tbody>
</table>

\(B_{\text{max}},\) apelin receptor density; K\textsubscript{D}, dissociation constant

Having characterised the distribution and localisation of the apelin receptor and its ligands in both human and mouse tissues, I next want to see how this translates to disease states. Notably, chronic kidney disease still represents a significant health burden globally and an area of unmet therapeutic need (Czopek et al., 2016). Recent studies have shown that the apelin system may be beneficial in acute kidney disease and possibly chronic kidney disease (Huang et al., 2018). To investigate potential changes in the apelin pathway in chronic kidney disease, the mouse model of ischaemia reperfusion-induced CKD and CKD patient plasma samples and control were obtained from Dr Dhaun, University of Edinburgh and analysed.

### 3.3.8 The Apelin System in Chronic Kidney Disease – Mice and Humans

Real-time quantitative PCR analysis of the left (diseased) and right (normal control) kidney showed a significant decrease in the expression of \textit{Apln} and \textit{Apela} mRNA in the diseased compared to control kidney (Fig. 3.22A-B). In addition, mRNA expression
of the receptor showed an upward trend but *Paired Student’s t-test* analysis did not show significance (Fig. 3.22C).

Figure 3.22. Expression of apelin system in the mouse model of chronic kidney disease A, *Apln* gene expression; B, *Apela* gene expression; C, *Aplnr* gene expression. * indicates *p*<0.05, ** indicates *p*<0.001, *** indicates *p*<0.0001. Data represent mean±SEM.

Next, plasma levels of apelin and ELA in chronic kidney disease patients were measured using an ELISA. It was observed that compared to control; there was a significant upregulation of apelin in the plasma of diseased patients compared to control (Fig. 3.23A). However, there was no difference between the plasma levels of ELA in diseased patients compared to control (Fig. 3.23B). When these data were sorted based on estimated glomerular filtration rate (eGFR), which is a measure of disease severity, there was a significant upregulation of plasma apelin in patients with
eGFR <60 ml/min/1.73m² compared those with eGFR >60 ml/min/1.73m² (6.87±0.69 vs 4.15±0.31 pg/ml, p<0.0001). Like apelin, the levels of ELA in the plasma of chronic kidney disease patients (eGFR <60 ml/min/1.73m²) were also significantly increased compared to patients with eGFR >60 ml/min/1.73m² (13.03±1.82 vs 8.49±0.97 pg/ml, p<0.05).

Furthermore, plasma levels of ELA in control patients were significantly higher than that of apelin (9.5±2.4 pg/ml ELA vs 3.78±0.5 pg/ml apelin) (Fig. 3.24A). A similar trend was also observed in chronic kidney disease patients (9.95±0.9 pg/ml ELA vs 5.39±0.4 pg/ml apelin) (Fig. 3.24B). Additionally, although there was no significant correlation between plasma levels of apelin and ELA in control patients (Fig. 3.24C), a significant positive correlation (r = 0.246, p<0.05) was observed between plasma levels of ELA and apelin in chronic kidney disease patients (Fig. 3.24D).
Figure 3.24. Comparison of apelin and ELA levels in control and CKD patient plasma. A, plasma levels of ELA compared to apelin in control patients; B, plasma levels of ELA compared to apelin in chronic kidney disease patients; C, correlation analysis for ELA and apelin in control patient plasma; D, correlation analysis for ELA and apelin in chronic kidney patient plasma. * indicates p<0.05, ** indicates p<0.001, *** indicates p<0.0001. Data represent Mean±SEM.
3.4 Discussion

The apelinergic system has emerged as an essential system in the regulation of cardiovascular function with a suggestive role in fluid homeostasis. ELA is a novel member of the apelinergic system initially discovered as a developmental peptide pivotal to normal cardiac development in zebrafish (Chng et al., 2013). Its functions in the adult mammalian system remain mostly unknown. Therefore, since the apelinergic system functions mainly in a paracrine/autocrine manner, the identification of tissues or cells expressing the receptor and ligands, especially the newly discovered ELA is critical to understanding its physiological functions in vivo. Using a combination of immunohistochemistry, confocal microscopy, autoradiography and RT-qPCR techniques, this study comprehensively characterised the distribution of the apelinergic system in human and mouse tissues. A novel distribution of ELA, apelin and apelin receptor in the renal nephron was identified. This study also reports for the first time, the expression of ELA in the mouse and human brain, kidney, lungs, left and right ventricle of the heart.

Interestingly, ELA mRNA was expressed at several folds high in mouse kidney compared to apelin or apelin receptor, but there was no difference between its levels and levels of apelin or apelin receptor in humans. In human kidney, the apelinergic system was expressed from the glomerulus to the collecting duct and with intense localisation of the apelin receptor protein in the juxtaglomerular apparatus. Apelin appeared to be localised mainly to the apical membrane of tubular epithelial cells whilst ELA, and apelin receptor was localised to both apical and basolateral membrane.

3.4.1 Mouse

To further characterise the distribution of the apelinergic system, Apela knockout and heterozygous as well as wildtype mice were obtained and used for the tissue-specific analysis of apelin, ELA and apelin receptor mRNA expression. So far, Medhurst et al.,
(2003) is the only study to have reported the distribution of apelin and apelin receptor in mice, although both ligand and receptor expression in the rat has been studied extensively by several groups. However, the distribution of ELA in the mice has not been reported previously. This study expands on previous studies to demonstrate that whilst the apelin receptor was expressed in the mouse heart, spleen, brain and lung; apelin and ELA expression were found in the kidney, heart and lung but not liver and spleen. In addition, Deng et al., (2015) previously suggested that in rats, ELA was exclusively expressed in the kidney whilst apelin and the receptor were more widely distributed.

In contrast, this study found that although ELA expression in the mouse kidney was over 80-fold higher than apelin receptor and nearly 40-fold higher than apelin, it was also expressed in the brain and heart (albeit at much lower levels compared to apelin or apelin receptor). Perjés et al., (2016) also found expression of ELA mRNA in the heart and showed that its levels were significantly higher in the non-cardiomyocytes (endothelial cells and fibroblast) compared to cardiomyocytes. Thus, the high level of ELA in the kidney may suggest that the kidney was the primary site of its production and secretion at least in rodents.

It was previously shown that the loss of apelin resulted in the downregulation of its receptor, the apelin receptor (Sato et al., 2013). In this study, there was a trend for decreased apelin but not the apelin receptor expression in Apela deficient brain whilst although the levels of apelin and apelin receptor appeared to have increased in Apela null heart, this was not significant. Interestingly, in the kidney where Apela was the most highly expressed gene in wildtype mice; there was a significant decrease in the levels of apelin but not the apelin receptor when compared to wildtype. This may be consistent with a differential/tissue-specific role of ELA signalling in different tissues and could support the possibility that apelin might be able to compensate for the loss of ELA in some tissues (heart). Although the loss of Apela in mouse placenta increased apelin expression, apelin was unable to rescue the developmental defects of Apela null placentas (Ho et al., 2017), supporting activation of differential downstream
signalling cascades by apelin and ELA. In experimental septic shock model, not only was ELA more protective than apelin, but ELA infusion significantly enhanced apelin protein and mRNA expression as well as apelin receptor mRNA in the kidney of these mice (Coquerel et al., 2017). However, ELA infusion did not affect cardiac apelin or apelin receptor expression in these mice, suggesting that although but apelin and ELA signal via the same receptor, they could activate different downstream pathways and also exhibit tissue-specific activity.

The expression of apelin and apelin receptor observed here agreed with other studies were both ligand and receptor mRNA was found in the mouse heart, kidney, brain and lungs (Hosoya et al., 2000; Medhurst et al., 2003). However, although the apelin receptor was expressed in the spleen and liver, apelin was absent in these tissues (Medhurst et al. 2003; Hosoya et al. 2000). In addition, Kawamata et al., (2001) did not detect apelin mRNA in the spleen or liver by RT-qPCR, but their enzyme immunoassay detected the peptide in these tissues, suggesting that mRNA levels may not necessarily reflect protein levels if mRNA levels are below the sensitivity of the assay used. Moreover, although present in the mouse, the apelin receptor appears to be absent in the rat liver and spleen (Medhurst et al. 2003; Hosoya et al. 2000), suggesting a potential species differences in the expression profile within rodents (Pope et al., 2012).

### 3.4.2 ELA-Apelin Receptor Signalling and the Renin-Angiotensin System

The renin-angiotensin system plays a pivotal role in the pathogenesis of cardiovascular disease. Angiotensin II (Ang II) signalling at the angiotensin receptor type 1 promotes pathological cardiac remodelling, including cardiac hypertrophy, fibrosis, inflammation and hypertension (Zablocki and Sadoshima, 2011). Angiotensin-converting enzyme 2 (ACE2) is a monocarboxypeptidase that converts Ang-II to Ang 1-7, which counteracts the pathological effects of Ang-II/AT1R signalling possibly via the Mas G protein-coupled receptor (Sato et al., 2013). Hence, ACE2 was suggested
to act as a negative regulator of Ang-II signalling in the failing heart. Interestingly, an antagonistic relationship was proposed between the apelin signalling pathway and the Ang-II/AT1R signalling pathway (Siddiquee et al., 2013). Thus, in aged mice deficient for apelin gene, ACE2 expression was significantly downregulated, and these mice went on to develop heart disease which was reversed by genetic and pharmacological inhibition of AT1R (Sato et al., 2013; Zhang et al., 2017).

Additionally, the beneficial effects of exogenous ELA in transaortic constriction model of pressure-overload induced heart failure was suggested to involve downregulation of ACE but not ACE2 mRNA and protein expression in mice (Sato et al., 2017). This means that the cardioprotective effects of apelin and ELA in heart failure may involve differential effects on ACE and ACE2 expression in mice. In support, it was observed in this study that although within the heart there was no difference between ACE and ACE2 levels in wildtype, ACE expression was significantly upregulated in Apela deficient and heterozygous heart compared to ACE2. However, there was also no significant change in cardiac ACE/ACE2 ratio, which was previously shown to positively correlates with mean arterial blood pressure, proteinuria and serum creatinine and is often used as a surrogate for activation of RAS in pathological states (Soler et al., 2013). This may further support a potential cardioprotective role of the ELA-apelin receptor pathway in mice.

Majority of studies investigating the interaction between RAS and apelinergic pathway have focused on the heart, and very little is known on renal changes. However, the pivotal link between the cardiovascular and renal system implies kidney diseases also affect the heart and contribute to cardiovascular defects. In this study, ACE2 expression was significantly upregulated in the wildtype and heterozygous kidney when compared to ACE, but there was no difference between ACE2 and ACE expression in Apela deficient mice. Notably, ACE/ACE2 ratio was significantly upregulated in Apela null kidney, which may suggest an upregulation of the renal RAS activity in the absent of ELA. This is consistent with the previously observed downregulation of ACE following ELA infusion in a mouse model of heart failure (Sato
et al., 2017) that in the absence of ELA ACE expression was upregulated which may result in increased angiotensin II levels. This increased ACE expression is a common feature of human cardiorenal diseases including diabetic nephropathy, chronic kidney disease, hypertension and heart failure (Mizuiri and Ohashi, 2015). A recent study demonstrated that intrarenal ELA signalling at the apelin receptor inhibited renin-angiotensin system to reduce blood pressure and protect against kidney injury (Xu et al., 2020). Taken together, renal loss of ELA signalling may exacerbate RAS signalling resulting in hypertension and renal injury mediated by the increased expression of ACE relative to ACE2 and possibly increased angiotensin II signalling.

3.4.3 Gene Expression Profile of the Apelinergic System in Humans

Previous studies on the distribution of the apelinergic system in human and animal tissues, including the kidney, have focussed on whole tissues. However, it is unclear whether there was a differential expression of the apelinergic system in different regions of the kidney, this study sought to examine the distribution of the apelinergic system in the two major renal regions, cortex and medulla but found no changes in expression. Within either the cortex or medulla, there was also no significant difference in ELA levels, compared to apelin or apelin receptor. Although apelin and apelin receptor mRNA were previously reported in human kidney (Medhurst et al. 2003), the distribution of ELA mRNA in other human tissues is unknown. This study is the first to confirmed the expression of ELA in the human brain. Low levels of ELA mRNA were previously detected in the endothelium of human heart and lungs (Yang et al., 2017b). ELA mRNA was also observed in human kidney and prostate (Chng et al., 2013; Wang et al., 2015), but these studies were not quantitative. Consistent with these reports, the level of ELA in the heart was significantly lower than that of the receptor and apelin, but in the lungs, there was no difference in the expression of ELA and apelin; although ELA level was significantly lower than apelin receptor. Furthermore, ELA levels in the brain and left and right ventricle of the heart were substantially lower than either apelin
or apelin receptor. This may be consistent with the endothelium and kidney being potential sources of ELA.

The right and left ventricle of the heart are functionally different, and both apelin and its receptor are widely expressed in the heart. However, the expression profile in the left relative to right ventricle has not been previously described. In this study, there was no difference in the levels of either apelin or apelin receptor in the left ventricle compared to the right ventricle of the human heart. However, ELA expression was significantly upregulated in the left compared to the right ventricle. The functional implications of these are unclear given that the role of ELA in cardiac physiology has not been fully explored. However, such differential gene expression pattern has previously been reported in the human (Su et al., 2015) and rat heart with signal transduction, apoptosis, cell growth and maintenance genes showing the most difference (Chugh et al., 2003). Why apelin or apelin receptor expression was not differentially upregulated or downregulated in this manner is unclear and may suggest an essential role of ELA in the left ventricle.

In a mouse model of LAD-induced myocardial infarction, both ELA and apelin receptor were upregulated in the left ventricle of infarcted hearts, and this was positively correlated with better preservation of left ventricular systolic function (Perjés et al., 2016). However, there was neither a change in apelin levels or association with left ventricular function in the infarcted heart (Perjés et al., 2016), consistent with a critical role for ELA in the regulation of cardiac physiology. Interestingly, the authors did not find any association between ELA and apelin receptor in sham-operated hearts, suggesting that the observed association in infarcted hearts may be an adaptive response to protect the diseased myocardium. In support Chapman et al., (2020) observed that circulating ELA in chronic kidney disease patients was positively associated with measures of cardiovascular risk but not renal function, and this may be an adaptive response to the endothelial dysfunction in these patients. Additionally, apelin is downregulated in severe myocardial infarction (Chong et al., 2006; Iwanaga et al., 2006). Taken together, the ELA-apelin receptor signalling pathway may play a
crucial role in the initial adaptive response to restore left ventricular function in the failing heart, consistent with a cardioprotective effect in heart failure.

3.4.4 Cellular Distribution of the Apelinergic System in Human Kidney

The results of this study showed that both ELA and apelin were expressed in the renal endothelial cells. This was consistent with previous studies, which revealed by immunohistochemistry that apelin was localised to vascular endothelial cells lining blood vessels in the kidney, heart and lungs (Kleinz and Davenport, 2004). Recently, Yang et al., (2017) showed that ELA, like apelin, was an endothelial peptide localised to endothelial cells in the human cardiac and pulmonary arteries with very little or no expression in the vein. Also, the localisation of the apelin receptor to the renal smooth muscle and endothelial cells in this study was in consonance with a previous report were similar observations were made in the heart (Kleinz et al., 2005), supporting the known critical role of the apelinergic system in the regulation of vascular function (Read et al., 2019a).

Apelin infusion in rats caused relaxation of angiotensin II pre-constricted afferent and efferent renal arterioles (Hus-Citharel et al., 2008), suggesting a potential role in renal blood pressure regulation. At the renal level, one of the mechanisms of blood pressure regulation involves secretion of renin by juxtaglomerular apparatus, which converts angiotensinogen to angiotensin I before the latter is further converted into the potent vasoconstrictor, angiotensin II. Although the apelin receptor is known to interact with the renin-angiotensin system, expression of the apelin receptor in the juxtaglomerular apparatus has not been previously reported. This study showed that apelin receptor was expressed in the juxtaglomerular apparatus. The localisation in this segment of the nephron was confirmed using a specific marker of the distal convoluted tubule, TRPM6. This may suggest a potential role of the apelin system in the regulation of systemic blood pressure. Additionally, the colocalisation of the apelin receptor with NKCC2, a key channel involved in the regulation of blood pressure and glomerular
filtration rate via tubuloglomerular feedback mechanism (Castrop and Schießl, 2014), further supports this hypothesis. Therefore, these findings may indicate that apelin/ELA may regulate glomerular haemodynamics and systemic pressure via a direct action on apelin receptors on the juxtaglomerular cells.

The observation that apelin, ELA and apelin receptor was expressed in the human glomerulus but possibly not in glomerular endothelial cells as evidenced by lack of colocalisation with von Willebrand factor was somewhat surprising. This is because both ligands are widely expressed in the endothelium and have been considered endothelial peptides (Yang et al., 2017b). However, consistent with these data, a recent study found apelin receptor immunoreactivity colocalised with various podocyte markers including nephrin and synaptopodin but not the endothelial marker, platelet-endothelial cell adhesion molecule 1 (PECAM-1), suggesting podocyte but not an endothelial expression in the glomerulus (Müller et al., 2018). Therefore, this may indicate a potential role of the apelinergic system in the regulation of podocyte function at the glomerulus level.

In the renal nephron, the glomerulus is connected to the rest of the nephron by the proximal convoluted tubule comprising primarily of epithelial cells. Although the expression of the apelin receptor mRNA in the proximal tubule was suggested (Hus-Citharel et al., 2008), apelin and ELA have not been previously characterised either at the mRNA or protein level in this region. Interestingly, this study showed that both ligands and their receptor were expressed in the epithelial cells of the proximal tubule, suggesting a potential autocrine function. Additionally, although both the apelin receptor and ELA appeared to be expressed in the apical and basolateral membrane, apelin appeared to be localised only to the apical membrane. The apical localisation of apelin and ELA does not only suggest a potential autocrine/paracrine role in the kidney but also suggest that they may be present in urine. Indeed, the urinary secretion of ELA peptides has been reported where a significant increase in secretion was found in the second and third trimester of pregnancy in women (Zhou et al., 2019). However,
it is not clear what the role of ELA in urine is although both ELA and apelin have been shown to promote diuresis (Deng et al., 2015; Hus-Citharel et al., 2008).

Furthermore, the colocalisation of apelin receptor immunoreactivity with SGLT2, an essential glucose transporter, did confirm not only proximal tubule expression but also suggest a potentially novel role for the apelin receptor in the regulation of renal glucose homeostasis. Interestingly, apelin increased glucose flux through gastric epithelial cells into the bloodstream by activating AMPKα2 and increasing the ratio of GLUT2/SGLT1 protein expression in mouse small intestine (Dray et al., 2013). Recently, Fournel et al., (2015) showed that luminally-released apelin is transcytosed into intraduodenal structures where it decreased enteric nervous system (ENS)-induced duodenal contraction. This resulted in increased glucose tolerance and insulin release via the hypothalamic release of ENS neurotransmitters acetylcholine and nitric oxide. The authors observed that increased glucose utilisation in the muscle was mediated by increased mRNA expression of GLUT2, suggesting that apelin stimulated glucose uptake and utilisation in the skeletal muscle. Therefore, the co-expression of the apelin receptor with SGLT2 in proximal convoluted tubule epithelial cells may suggest the involvement of apelin signalling in the regulation of glucose flux at the renal level as described in the intestine.

Like the proximal convoluted tubule, the thin descending limb of the loop of Henle is vital for the maintenance of fluid and electrolyte homeostasis in the body (Curthoys and Moe, 2014; Pannabecker, 2012). However, the expression of apelin, ELA or apelin receptor in this segment of the nephron has not been previously described. This study showed that both ligands and receptor were expressed in the LoH, suggesting a potential autocrine/paracrine role. It is not clear what the function of the apelinergic system in this region might be hence further studies are required to explore this. Similarly, apelin, ELA and apelin receptor was also localised to the thick ascending limb of the loop of Henle (TAL). Consistent with these observations, the apelin receptor mRNA has been reported in both cortical and medullary TAL (Hus-Citharel et al., 2008). Still, the expression of apelin and ELA observed in this study is novel and have
not been observed previously. Although the role of apelin signalling in this region is not known, it may be involved in the regulation of NKCC2 cell-surface trafficking and function. The cell-surface trafficking of NKCC2 is regulated by hormonal signalling pathways including the stimulatory cAMP-dependent pathway mediated by angiotensin II, vasopressin and glucagon or the inhibitory cGMP-dependent pathways mediated by nitric oxide, ANP and endothelin-1 (Castrop and Schießl, 2014). Apical localisation of AQP2 in the collecting duct is regulated by vasopressin-dependent cAMP accumulation. However, apelin signalling inhibits cAMP release and AQP2 expression in the apical membrane resulting in increased diuresis (Hus-Citharel et al., 2014). Therefore, it is plausible that a similar mechanism may regulate apical translocation of NKCC2 in TAL. However, further studies may investigate the effect of apelin signalling in TAL on NKCC2 trafficking.

Apelin, ELA and apelin receptor expression colocalised with aquaporin 4 in the collecting duct supporting an autocrine role of the apelinergic system in the regulation of water reabsorption in this region of the nephron (Hus-Citharel et al., 2014). Consistent with a role in fluid homeostasis, water deprivation or salt loading in humans and rodents resulted in an inverse regulation of vasopressin and apelin expression to allow the systemic release of vasopressin (Azizi et al., 2008; Goazigo et al., 2004), thereby preventing additional water loss from the kidney. Apelin and vasopressin were colocalised in magnocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) (Reaux et al., 2002), where apelin was suggested to inhibit vasopressin release and neuronal activity (De Mota et al., 2004). Furthermore, apelin receptor expression did not only colocalised with vasopressin receptor 1 (V1R) and vasopressin in the PVN but also mice deficient in apelin receptor could not concentrate urine (Flahault et al., 2017; Goazigo et al., 2004). Central administration of ELA activated vasopressinergic neurons in the PVN and supraoptic nucleus of the hypothalamus (Santoso et al., 2015). Taken together, this supports the role of the apelinergic system in the modulation of fluid homeostasis at the renal and central nervous system. Hence, targeting the apelinergic system may be a potential
therapeutic strategy in the management of water handling disorders such as hyponatremia and polyuria-polydipsia syndrome (Flahault et al., 2017).

3.4.5 Potential Species-Difference in Apelin Receptor Expression

Immunohistochemistry, although a useful technique for characterising the cellular distribution of proteins in cells and tissues is limited by its inability to provide information about the functional state of receptor proteins. Therefore, to describe the distribution of apelin receptor binding sites in the kidney, receptor autoradiography was performed using $^{125}\text{I}$apelin-13 in both mouse and human kidneys. Interestingly, in human kidneys, dense distribution of $^{125}\text{I}$apelin-13 binding sites were observed in both renal cortex and medulla. In the mouse kidney, a dense localisation of $^{125}\text{I}$apelin-13 binding site was found in the mouse outer medulla, but no apparent evidence of binding was observed in the cortex. Whilst this may highlight a potential species-difference in apelin receptor distribution in human compared to mouse kidney, the possibility that there could be sparse binding sites in other regions of either the human or mouse kidney that were below the level of detection by this technique cannot be excluded. Consistent with these data, Pope et al., (2012) used autoradiography to show that apelin receptor was localised to mouse renal medulla, lungs and heart. The identification of $^{125}\text{I}$apelin-13 binding sites in the apelin receptor suggests that the receptor is correctly processed and folded. However, it does not unquestionably infer that the receptor is capable of signalling.

3.4.6 The Apelinergic System in Chronic Kidney Disease

Chronic kidney disease characterised by elevated blood pressure, vessel stiffness and heart disease affect millions of people in the UK, but there is no available cure except kidney transplantation (Czopek et al., 2016). Although the apelin pathway has been suggested as a potential therapeutic target for the treatment of CKD, very little is known about the expression and function in diseased kidney. Here, it was shown using a rodent model of CKD that although the presence of both ligands of the apelin
receptor is significantly downregulated, the receptor was unaffected. This suggests that the apelinergic system may be a novel therapeutic target for the treatment of this disease and that exogenous apelin or ELA may compensate for the decreased endogenous expression of these ligands and confer renoprotection against CKD. Indeed, exogenous administration of either apelin or ELA in animal models of CKD were shown to attenuate decrease progression by inhibiting apoptosis and improving renal function (Chen et al., 2017; Czopek et al., 2016).

Despite the suggested renoprotective role of the apelinergic system, very little is currently known about the ligands and their receptor function in human disease. Here, increased circulating apelin and ELA were found in the plasma of CKD patients. In addition, a modest positive correlation was observed between the circulating apelin and ELA in these patients, which may be an adaptive response to endothelial dysfunction. Malyszko et al., (2008) found a decrease in plasma apelin in kidney transplant recipients with underlying coronary artery disease (CAD) compared to those without CAD, and this was associated with endothelial damage and inflammation. In diabetic nephropathy patients, ELA levels were decreased as the levels of serum albumin and creatinine increased (Zhang et al., 2018a). It has also been reported that apelin levels were reduced in diabetic nephropathy, but patients with high apelin levels have better survival rates, with decreased risk of cardiovascular mortality when compared to those with lower apelin levels (Silva et al., 2013). However, in patients with autosomal dominant polycystic kidney disease (ADPKD) progressing to end-stage renal disease, apelin levels decreased substantially (Kocer et al., 2016). Importantly, in ADPKD, a low level of apelin was an independent predictor of renal disease progression and marker of renal replacement therapy response (Lacquaniti et al., 2013). Taken together, this may support a renoprotective role for the apelinergic system in chronic kidney disease.
3.4.7 Conclusion and Further Work

In conclusion, this study reports a novel distribution of the apelinergic system, particularly ELA in mouse and human tissues (Table 3.4). This included novel expression of ELA in the brain, lungs, left and right ventricle of the heart in humans and the mouse brain and heart. There was no evidence of differential expression of components of the apelinergic system in the kidney and considering the robust distribution of apelin receptor binding sites in human kidney, where the apelin system is least studied further characterisation of this tissue was performed. Interestingly, apelin, ELA and apelin receptor were localised in renal blood vessels, glomerulus as well as epithelial cells of the nephron through to the collecting duct. Additionally, strong expression of the apelin receptor was detected in juxtaglomerular cells, indicating a potential role of the apelinergic system in control of systemic blood pressure. This study suggests that the apelinergic system may have a crucial role in the regulation of both renal physiology and pathophysiology and could present a novel therapeutic target for the treatment of kidney diseases.

Table 3.4. Summary of the expression mRNA expression profile of apelin, apelin receptor and ELA (Apela) in mouse and human tissues.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Apelin mRNA</th>
<th>Apelin receptor mRNA</th>
<th>ELA mRNA</th>
<th>(Apela)</th>
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<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>Very high</td>
<td>High</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>Moderate</td>
<td>Very high</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>Low</td>
<td>Low</td>
<td>Very high</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>Not detected</td>
<td>High</td>
<td>Not detected</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Not detected</td>
<td>High</td>
<td>Not detected</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td></td>
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<tr>
<td>----------------</td>
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<td>---------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td><strong>Humans</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Brain</strong></td>
<td>Moderate</td>
<td>Very high</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td><strong>Lung</strong></td>
<td>Moderate</td>
<td>Moderate</td>
<td>Moderate</td>
<td></td>
</tr>
<tr>
<td><strong>Heart</strong></td>
<td>Moderate</td>
<td>Very high</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td>Moderate</td>
<td>Moderate</td>
<td>Moderate</td>
<td></td>
</tr>
</tbody>
</table>

### 3.4.7.1 Further Work

This study found expression of the apelin system, especially the newly discovered peptide ELA, in several tissues in both mice and humans. However, within the scope of this thesis, only the renal expression was characterised in detail. Considering that some of the organs such as brain have complex and physiologically different anatomical regions, further work will be required to characterise the experience of ELA in the brain particularly, the hypothalamus where apelin and apelin receptor expression has been described previously. For instance, apelin and apelin receptor expression at the protein level has been reported in the PVN and SON, where they colocalised with vasopressin (Reaux-Le Goazigo et al., 2004; De Mota et al., 2004). It is was worth noting that the brain also has vascular component and although both apelin and ELA were suggested to be endothelial peptides (Yang et al., 2017), it is not clear whether magnocellular neurons in the PVN and SON or endothelial cells of the brain are the main source of ELA or apelin, and this could be clarified in further studies. Within the kidney, further studies could investigate the role of apelin receptor system in the juxtaglomerular apparatus, and whether loop diuretics may affect circulating levels of apelin or ELA in patients.
Chapter 4 Identification of Endogenous ELA Peptides and ELA-32 Metabolites in Human Plasma and Kidney Homogenates by Mass Spectrometry

4.1 Introduction

4.1.1 Background

Elabela/Toddler (ELA) was identified in a region of the genome previously thought to not encode a functional gene as the second endogenous ligand of the apelin receptor and shown to be important for proper heart development in zebrafish (Chng et al., 2013; Pauli et al., 2014). ELA is translated as a 54 amino acid preproprotein, which undergoes proteolytic processing to form mature ELA comprising 32 amino acids. Earlier studies predicted the existence of two other isoforms of the peptide namely ELA-21 and ELA-11, based on the presence of dibasic cleavage sites in the ELA-32 peptide sequence (Fig. 4.1) (Chng et al., 2013; Pauli et al., 2014). The Davenport group and others have shown that all three isoforms were active at the apelin receptor (Pauli et al., 2014; Yang et al., 2017b). Furthermore, another isoform of the peptide, ELA-22 have previously been reported in vitro in rodent plasma (Murza et al., 2016). However, experimental evidence for the generation of these isoforms in human plasma or tissue is lacking, and it remains unknown which isoform is abundantly produced endogenously, and thus would be relevant as a tool compound for studying ELA signalling.
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4.1.2 The Need for Mass Spectrometry

Presently, determination and quantification of ELA peptides in human fluids and tissues are done mostly by immunological methods, and this is based on the recognition of the shared C-terminal fragment. Although these methods are very robust, they cannot discriminate between the different ELA isoforms without any prior chromatographic separation as previously used for apelin isoforms by Maguire et al., 2009. When this chromatographic separation is coupled with mass spectrometry, it is a very sensitive technique to identify and quantify individual isoforms of a protein or peptide.
4.1.3 Hypotheses and Aims

It was hypothesised that ELA-21 and ELA-11 would be the principal metabolites of ELA-32 and that one of these isoforms would be produced abundantly in the kidney. Thus, a tandem mass spectrometry-based assay was developed to detect ELA derived peptides and to show whether ELA-21 and ELA-11 or ELA-22 could be generated proteolytically in vitro in human plasma and kidney homogenates, and identify the most abundant endogenous isoform. ELA-11, ELA-16, ELA-18, ELA-19 and ELA-22 were identified as fragments of ELA-32 generated in human plasma. Also, ELA-11 was identified as the potential most abundant isoform of ELA peptides produced endogenously.
4.2 Materials and Method

4.2.1 Materials

All materials used in this chapter were listed in Chapter 2, section 2.1. All other reagents were obtained from Sigma.

4.2.2 Peptide Extraction from Human Tissues using Guanidine Hydrochloride

Peptides were extracted from human tissues as previously described (Roberts et al., 2019) and detailed in section 2.8.1.2. Peptides were subsequently reduced and alkylated and stored at -20 °C until analysis on LC-MS/MS.

4.2.3 Triple Quadrupole LC-MS Analysis of ELA Peptides

Peptide extracts (15 µl) were injected onto a Waters UPLC H-Class LC system (Waters, Milford, MA) using a 2.1 × 50 mm 1.8 mm particle HSS T3 Acquity column as described in section 2.8.1.1. This technique is very robust and sensitive for quantification of known analytes (please see Chapter 2, section 2.7 for a detailed description of the principle). The transitions of ELA peptides monitored were listed in table 4.1 below.
Table 4.1. SRM transitions of ELA peptides monitored on the triple quadrupole mass spectrometer.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Charge state</th>
<th>Precursor m/z (Q1)</th>
<th>Collision energy eV (Q2)</th>
<th>Product m/z (Q3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELA 32 (transition 1)</td>
<td>7</td>
<td>581.84</td>
<td>18</td>
<td>659.5</td>
</tr>
<tr>
<td>ELA 32 (transition 2)</td>
<td>7</td>
<td>581.84</td>
<td>20</td>
<td>654.78</td>
</tr>
<tr>
<td>ELA 32 (transition 3)</td>
<td>7</td>
<td>581.84</td>
<td>20</td>
<td>116.0</td>
</tr>
<tr>
<td>ELA 21 (transition 1)</td>
<td>6</td>
<td>451.44</td>
<td>18</td>
<td>562.09</td>
</tr>
<tr>
<td>ELA 21 (transition 2)</td>
<td>6</td>
<td>451.44</td>
<td>13</td>
<td>512.75</td>
</tr>
<tr>
<td>ELA 11 (transition 1)</td>
<td>3</td>
<td>447.69</td>
<td>13</td>
<td>613.31</td>
</tr>
<tr>
<td>ELA 11 (transition 2)</td>
<td>3</td>
<td>447.69</td>
<td>13</td>
<td>525.28</td>
</tr>
<tr>
<td>ELA 11 (transition 3)</td>
<td>3</td>
<td>447.69</td>
<td>15</td>
<td>115.94</td>
</tr>
</tbody>
</table>

Q1, quadrupole 1 (mass filter); Q2, quadrupole 2 (for collision-induced dissociation and fragmentation of precursor ion), Q3, quadrupole 3 (mass filter for product ions)

4.2.4 Peptide Extraction for Immunoprecipitation

Human kidney (n=5; 193-241 mg), normal brain (n=5; ~300mg), glioblastoma (n=4; ~300mg) and coronary artery (n=7; 93-173 mg) samples were boiled at 100 °C for 20 mins and extracted (section 2.9.5). An 8-point calibration line (10 pg/ml-50,000 pg/ml) was generated using 0.1% BSA as surrogate matrix from a stock (10 µg/ml) of ELA-32, -21 and ELA-11 all mixed together. Extracted samples and 150 µl of each standard were evaporated, reduced and alkylated, and 20 µl injected into the LC-MS/MS system for analysis.
4.2.5 Enzyme-based Immunosorbent Assay (ELISA)

The normal human brain (n=8) and glioblastoma (n=9), and samples of extracted and immunoprecipitated peptides recovered after performing LC-MS/MS analysis; evaporated to remove formic acid and reconstituted in assay buffer were analysed in the ELISA assay. The ELISA assay was performed following the manufacturer’s instructions, as described in section 2.10.

4.2.6 ELA Degradation in Human Plasma and Human Kidney Homogenate

Human plasma samples obtained from 4 healthy individuals (3 males, 1 female) and human kidney homogenates (n=3) were pooled together (Section 2.8.1.7). ELA-32 (5 \( \mu \)g/ml final concentration) was incubated at 37 °C and aliquots (plasma 50 \( \mu \)l; homogenate 100 \( \mu \)l) withdrawn at different time-points (plasma: 0, 2, 5, 10, 15, 30, 60, 120, 240; homogenate: 0, 2, 5, 10, 15, 30, 60, 120); and mixed with half volume ice-cold guanidine hydrochloride (6 M). Samples were extracted and analysed on ThermoScientific Ultimate 3000 LC system connected to a ThermoScientific Orbitrap Q-Exactive Plus mass spectrometer in high flow using 2.1 x 50 mm 1.8 mm particle HSS T3 Acquity column (Section 2.8.1.8) or nanoflow using the build-in C18 column (Section 2.8.1.9)

4.2.7 Peptide Identification Using PEAKS Software

Raw LC-MS/MS data files were searched through PEAKS X software as described in Section 2.8.3. FDR was set to 1% against a decoy database, and a precursor and product ion tolerances were set as 10 ppm and 0.05 m/z respectively.

4.2.8 Data Analysis

Peak areas were quantified using the Qualbrowser software (ThermoFisher, San Jose, CA) and expressed as mean±SD, as a standard procedure. Unknown ELA
concentrations measured by ELISA were determined by interpolation to a standard curve of known concentrations fitted to a 4-parameter logistic concentration-response curve in GraphPad Prism 6. Data were expressed as mean±SEM.
4.3 Results

4.3.1 Stability of ELA-32 in Human Plasma and Kidney Homogenates

In human plasma, ELA-32 slowly degraded such that over 20% of the parent peptide was still detectable after 240 mins (Fig. 4.2A). However, degradation in kidney homogenates was very rapid, where no parent peptide was detectable after 20 mins (Fig. 4.2A), possibly due to the presence of several proteolytic enzymes. The half-life of ELA-32 in human plasma and kidney homogenates were 47.2±5.7 mins and 44.2±3 secs, respectively. In addition to the expected cysteine alkylation, several other modifications of the parent ELA-32 peptide were apparent, including mono-oxidation, di-oxidation and alkylation of lysine residues (referred to as over-alkylation throughout this thesis) (Fig. 4.2B), all of which are likely to be extraction artefacts.
Figure 4.2. Degradation profile of ELA-32 in human plasma and kidney homogenates assessed over four- and two hour-incubation periods, respectively. A, Half-life of ELA-32 in human plasma (red) and kidney homogenates (blue); B, ELA-32 and some of the ELA-32 side reactions observed in human plasma. The amount of ELA present gradually decreased with time. Data represent mean±SD.
4.3.2 Identification of ELA-32 Metabolites

To identify metabolites from ELA-32 in human plasma, a full scan analysis was first performed to understand the degradation profile of intact ELA-32 peptide in the high flow LC-MS/MS (less sensitive but very fast) on an Orbitrap mass spectrometer. However, database searching of the acquired MS/MS data failed to identify any metabolites. Therefore, selected samples were re-analysed using the more sensitive nano LC-MS/MS and a data-dependent acquisition (DDA) analysis. The LC-MS/MS data were analysed on PEAKS X software and compared against the UniProt protein database. The amino acid sequences, observed mass and mass accuracy (ppm) for the C-terminal and N-terminal metabolites of ELA-32 that were identified are shown in Table 4.2 and Table 4.3.
Table 4.2. ELA-32 fragments derived from the loss of C-terminal amino acid residues.

<table>
<thead>
<tr>
<th>C-terminal metabolites</th>
<th>Length</th>
<th>Mass</th>
<th>ppm</th>
<th>m/z</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELA-32</td>
<td>1-32</td>
<td>4096.16</td>
<td>0.8</td>
<td>513.03</td>
<td>8</td>
</tr>
<tr>
<td>C-terminal metabolites</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELA-32(_{1-31})</td>
<td>1-31</td>
<td>3967.12</td>
<td>9.2</td>
<td>441.80</td>
<td>9</td>
</tr>
<tr>
<td>ELA-32(_{1-21})</td>
<td>1-21</td>
<td>2758.53</td>
<td>0.4</td>
<td>460.76</td>
<td>6</td>
</tr>
<tr>
<td>ELA-32(_{1-20})</td>
<td>1-20</td>
<td>2602.43</td>
<td>1.2</td>
<td>434.74</td>
<td>6</td>
</tr>
<tr>
<td>ELA-32(_{1-19})</td>
<td>1-19</td>
<td>2487.35</td>
<td>-0.3</td>
<td>415.57</td>
<td>6</td>
</tr>
<tr>
<td>ELA-32(_{1-16})</td>
<td>1-16</td>
<td>2045.15</td>
<td>0.9</td>
<td>410.03</td>
<td>5</td>
</tr>
<tr>
<td>ELA-32(_{1-14})</td>
<td>1-14</td>
<td>1835.08</td>
<td>0.9</td>
<td>459.78</td>
<td>4</td>
</tr>
<tr>
<td>ELA-32(_{1-13})</td>
<td>1-13</td>
<td>1665.96</td>
<td>-0.8</td>
<td>417.49</td>
<td>4</td>
</tr>
<tr>
<td>ELA-32(_{1-12})</td>
<td>1-12</td>
<td>1509.85</td>
<td>-0.1</td>
<td>504.29</td>
<td>3</td>
</tr>
<tr>
<td>Other Metabolites generated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELA-19(_{1-17})</td>
<td>13-30</td>
<td>2204.10</td>
<td>3.1</td>
<td>1103.0</td>
<td>2</td>
</tr>
<tr>
<td>ELA-16(_{1-14})</td>
<td>16-30</td>
<td>1824.90</td>
<td>0.3</td>
<td>457.23</td>
<td>4</td>
</tr>
</tbody>
</table>

Mass, observed mass; ppm, parts per million (is a measure of mass accuracy); m/z, mass to charge ratio. ELA-19\(_{1-17}\) and ELA-16\(_{1-14}\) were derived from des-Pro\(^{17}\)-ELA-19 and des-Pro\(^{16}\)-ELA-16 respectively and maybe a substrate of ACE2.
Table 4.3. ELA-32 fragments produced from loss of N-terminal amino acid residues. Key fragments are shown in bold.

<table>
<thead>
<tr>
<th>N-terminal metabolites</th>
<th>Length</th>
<th>Mass</th>
<th>ppm m</th>
<th>m/z</th>
<th>charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELA-32 QRPVNLTMRRKLRKHNLQRRCMPLHSRVFP</td>
<td>32</td>
<td>4096.2</td>
<td>0.8</td>
<td>513.03</td>
<td>8</td>
</tr>
<tr>
<td>ELA-23 RKLRKHNLQRRCMPLHSRVFP</td>
<td>9-32</td>
<td>3042.6</td>
<td>0.5</td>
<td>508.11</td>
<td>6</td>
</tr>
<tr>
<td>ELA-22 KLRKHNLQRRCMPLHSRVFP</td>
<td>10-32</td>
<td>2845.5</td>
<td>2</td>
<td>475.26</td>
<td>6</td>
</tr>
<tr>
<td>ELA-20 RKHNCLQRRCMPLHSRVFP</td>
<td>12-32</td>
<td>2604.3</td>
<td>0</td>
<td>435.06</td>
<td>6</td>
</tr>
<tr>
<td>ELA-19 KHNCLQRRCMPLHSRVFP</td>
<td>13-32</td>
<td>2448.2</td>
<td>0.2</td>
<td>490.65</td>
<td>5</td>
</tr>
<tr>
<td>ELA-18 HNCLQRRCMPLHSRVFP</td>
<td>14-32</td>
<td>2320.1</td>
<td>0.8</td>
<td>465.03</td>
<td>5</td>
</tr>
<tr>
<td>ELA-17 NCLQRRCMPLHSRVFP</td>
<td>15-32</td>
<td>2183.1</td>
<td>-0.2</td>
<td>437.62</td>
<td>5</td>
</tr>
<tr>
<td>ELA-16 CLQRRCMPLHSRVFP</td>
<td>16-32</td>
<td>2069.0</td>
<td>0.6</td>
<td>414.81</td>
<td>5</td>
</tr>
<tr>
<td>ELA-11 CMPLHSRVFP</td>
<td>21-32</td>
<td>1355.6</td>
<td>0.3</td>
<td>452.89</td>
<td>3</td>
</tr>
<tr>
<td>ELA-9 PLHSRVFP</td>
<td>23-32</td>
<td>1048.6</td>
<td>2.2</td>
<td>525.30</td>
<td>2</td>
</tr>
<tr>
<td>ELA-8 LHSRVFP</td>
<td>24-32</td>
<td>951.53</td>
<td>1.3</td>
<td>476.77</td>
<td>2</td>
</tr>
</tbody>
</table>

Mass, observed mass; ppm, parts per million (is a measure of mass accuracy); m/z, mass to charge ratio.
4.3.2.1 Identification of ELA-32 metabolites in Human Plasma

In plasma, ELA-32 was cleaved to produce both N- and C-terminal metabolites. The most abundant metabolites produced from the loss of C-terminal amino acids (C-terminal metabolites) were ELA-32\(_{1-31}\), ELA-32\(_{1-14}\) and ELA-32\(_{1-12}\) (Table 4.2; Fig. 4.3A). The corresponding C-terminal fragments of some of these metabolites (N-terminal metabolites) were also identified, including ELA-18, ELA-20 and ELA-11 (Table 4.3; Fig. 4.3B). ELA-16, ELA-19 and ELA-22 were also present, but there was no evidence of ELA-21 previously thought to be produced from ELA-32 (Chng et al., 2013). Interestingly, ELA-16\(_{1-14}\) and ELA-19\(_{1-17}\) generated from the cleavage between the C-terminal Pro-Phe of the corresponding des-Pro\(^{16}\)-ELA-16 and des-Pro\(^{19}\)-ELA-19 peptides, respectively, which is suggestive of ACE2 activity was also identified (Table 4.2). Chromatograms of the most relevant metabolites, ELA-11 (previously proposed as a metabolite of ELA-32), and ELA-16 (mainly because of the disulphide bridge which will stabilise it against proteolysis) were shown in figures 4.4 and 4.5 respectively. The chromatogram of all other metabolites was shown in Appendix 1A-G.
Figure 4.3. ELA-32 metabolites identified from human plasma following nanoflow LC-MS/MS analysis. A, C-terminal metabolites generated as a product of cleavages at the C-terminus of ELA-32; B, N-terminal metabolites generated as a result of hydrolysis at the N-terminus of ELA-32. Selected samples were analysed in the nanoflow to identify metabolites that were produced during the incubation period (240 mins). ELA-21 (-48 Da) indicate ELA-21 with the loss of CH$_3$SH group corresponding to 48 Dalton (Da) from the methionine residue.
Figure 4.4. Representative chromatogram of ELA-11 (B) generated from ELA-32 in human plasma together with the corresponding N-terminal fragment ELA-32(1-21) (A). The metabolites were produced from the hydrolysis of mature ELA-32 between Arg²¹ and Cys²², but the hydrolytic enzyme is unknown. ELA-32(1-21), retention time 38.30 mins; ELA-11, retention time 43.76 mins, was only present in the oxidised form (methionine oxidation). The red stroke indicates the cleavage point.
Figure 4.5. Representative chromatogram of ELA-16 (B) generated from ELA-32 in human plasma together with the corresponding N-terminal fragment, ELA-32_{1-16} (A). ELA-32_{1-16} and ELA-16 were generated from the cleavage between Asn^{16} and Cys^{16}. ELA-32_{1-16}, retention time 32.65 mins; ELA-16 was only present in the oxidised form (methionine oxidation); retention time 40.80 mins. The red stroke indicates the cleavage point.
A. Degradation Profile of ELA-32 in Human Plasma

In order to elucidate the metabolite generation profile, the original full-time course dataset (obtained from the high flow full scan LC-MS/MS) was re-analysed and interrogated for metabolites by manual searching for the theoretical mass of the peptides identified in the nano LC-MS analysis. A major challenge to this approach was that the nanoflow analysis generated higher charge states for most of the identified peptides as nanospray is more efficient than high flow, and TFA reduced the efficiency further. The data showed that within the first 2 mins, ELA-16 and ELA-19 were generated from ELA-32; followed by ELA-8 and ELA-18 at 5 and 15 mins, respectively. All other fragments resulting from the loss of N-terminal amino acids were detected from 60 mins, where the most abundant metabolites were ELA-20, ELA-17, and ELA-11 (Fig. 4.6). The N-terminal fragment ELA-32(1-13), resulting from the cleavage that produced ELA-19 was also observed at the same time as ELA-19. However, ELA-9, ELA-21, ELA-22 and ELA-23 were not detected by this method.
Figure 4.6. Degradation profile of mature ELA-32 peptide in human plasma over a 240 mins incubation period. ELA-32\textsubscript{(1-13)} is the corresponding N-terminal fragment of ELA-19. All other isoforms were C-terminal fragments of ELA-32. Data represent mean±SD.

4.3.3 Identification of ELA-32 Metabolites in Kidney Homogenates

The same metabolite identification approach was used for the tissue homogenates. However, the initial high flow analysis did not identify any metabolites of ELA using the DDA analysis and peptidomics search strategy. In the subsequent nanoflow analysis, only two fragments resulting from the loss of N-terminal amino acids, namely ELA-23 and ELA-19, were identified in kidney homogenates (Fig. 4.7A). Interestingly, the corresponding C-terminal sequence of ELA-19, ELA-32\textsubscript{1-13} was also determined after manual searching. The parent peptide (ELA-32) levels decreased rapidly and were present in oxidised and overalkylated form (Fig. 4.7B).
Furthermore, the high flow LC-MS/MS data were re-analysed and searched for the metabolites that were identified in the plasma. Using this method, similar metabolites to those identified in the plasma were detected, however unlike the plasma were ELA-16 was the highest, ELA-18 was the most abundant in the kidney homogenate samples (Fig. 4.7C). Also, other fragments, including ELA-23, ELA-19 and ELA-16, were detected from the earliest time points. However, their levels decreased significantly with time, except for ELA-18, which was highest at 30 mins but was completely undetectable after 60 mins. ELA-17 was only detected in the last one hour of the incubation (60 mins and 120 mins samples), with the lowest level present at the latter time point. However, there was no evidence of ELA-21 in these samples, although ELA-11 was present.
Figure 4.7. ELA-32 metabolites identified from human kidney homogenates following nano and high flow LC-MS/MS analysis. A, Metabolites generated as a product of cleavages at the N-terminus of ELA-32 identified by nanoflow analysis; B, levels of modified ELA-32 peptides; C, Metabolites identified from manual searching of high flow data showing the timecourse of ELA-32 degradation. No C-terminal metabolites were identified in this case except ELA-32(1-13).
4.3.4 Development of Extraction Methods for Detection of Endogenous ELA Isoforms by LC-MS/MS

Having identified potential endogenous cleavage sites of ELA-32, I next wanted to identify the most abundantly produced endogenous ELA peptide. Previous studies have shown that the highest level of ELA mRNA expression was present in the human coronary artery (Yang et al., 2017b), while the kidney and prostate are other organs expressing the gene abundantly (Chng et al., 2013; Deng et al., 2015). Therefore, peptides were extracted from these tissues using a well-validated method for intact peptide extraction as previously described (Roberts et al., 2019), and analysed by LC-MS/MS. The resulting raw data files were put through Uniprot database searching for the presence of ELA peptides using PEAKS Studio X software. ELA peptides spiked into the positive controls were detected, but no endogenously derived ELA peptides were observed in either coronary artery, kidney or rat kidney. However, several other proteins or peptide fragments were detected. The molecular/functional interaction between these proteins was determined using the Search Tools for the Retrieval of Interacting Genes/Proteins (STRING) database (Fig. 4.8). The main proteins detected in the coronary artery were members of the histone family as well as structural proteins such as tubulins, actin and septin-9. Hepcidin-20, a protein involved in the absorption and transportation of iron in tissues (Nemeth and Ganz, 2009), was also identified. Other proteins identified included calmodulin 1-3, protein S100-A/B, and adipogenesis regulatory factor (ADIRF) isoforms, namely ADIRF-16, ADIRF-21, ADIRF-63 and ADIRF-64 (Appendix 1H).

In human kidney, 184 proteins/peptides were detected with RNA binding proteins and transcription factors showing the most substantial interaction in the network (Fig. 4.8). The most enriched pathways were mitochondrial proteins involved in the generation of metabolites and energy, including cytochrome c protein family, succinate CoA ligase and ATP-processing or related proteins including ATPases and ATP synthase. Structural proteins like tubulins, actin, vimentin, cofilin-1, spectrin beta-chain and
histones, as well as enzymes, including malate dehydrogenase, superoxide dismutase and corticosteroid 11-beta-dehydrogenase isozyme 2 were identified (Fig. 4.8). Other proteins detected include protein S100-A6, metallothionein-1G, peroxiredoxin-6, mitochondrial peroxiredoxin-5, glutaredoxin-3, calmodulin 1-3, Low-density lipoprotein receptor-related protein 2, Alpha-1-antitrypsin, hepatoma-derived growth factor and cystatin-B.

Given that rat kidney has been shown to have very high levels of ELA mRNA (Deng et al., 2015), peptides were also extracted from this tissue and run together with human samples on Orbitrap mass spectrometer. In these samples, there was no evidence of ELA, but several other peptides and proteins were identified (Appendix 1I). The most predominant group of proteins identified were enzymes including malate dehydrogenase, fructose-bisphosphate aldolase, ATP synthase, 6-phosphogluconate dehydrogenase decarboxylating, mitochondrial Succinyl-CoA:3-ketoacid coenzyme A transferase 1 and cathepsin L1. Ion channels and their regulators, including Na(+)/H(+) exchange regulatory cofactor (NHE-RF1), sodium/glucose cotransporter 2 and electrogenic sodium bicarbonate cotransporter 1 were also identified. Some structural proteins and receptors including moesin, vimentin, alpha-Parvin and ryanodine receptor 2 were present. Other proteins identified in rat kidney include cystatin-B, mitochondrial stomatin-like protein 2, vascular endothelial growth factor A (VEGFA), granulins and low-density lipoprotein receptor-related protein 2.
Figure 4.8. Protein-protein interaction networks for proteins identified in the human kidney. Interaction networks were generated using Search Tools for the Retrieval of Interacting Proteins/Genes (STRING) version 11. Each line represents the interaction between proteins that are likely to function in a similar pathway or have similar functions. In the red cycle are mitochondrial proteins cytochrome c oxidases (COX) and its subunits including COX8A, COX7C, NDFA4/5 and NDFS3. The type of protein interaction and their effects are colour-coded and shown on the bottom-left panel.
4.3.5 Enrichment of ELA Peptides in Extracted Samples by Immunoprecipitation

Antibody-based enrichment of peptide of interest following extraction is a well-characterised method for concentration of analytes in solution (Lee et al., 2016). Therefore, since ELA peptides were not detected using the first method, the next strategy I investigated was immunoprecipitation of tissue extracts with an antibody against ELA peptides. This antibody cross-reacts with all the proposed ELA isoforms including ELA-32, ELA-21 and ELA-11. The affinity of the antibody for all three isoforms was first assessed on the Triple quadrupole mass spectrometer (TQX-MS) using two types of beads: Dynabeads M-280 tosylactivated and Protein A/G magnetic beads coupled to either purified or unpurified antibodies at two different concentrations. Two concentrations of ELA, 5 ng/mL and 250 ng/mL representing lower and upper limits were tested. The Protein A/G coupled to unpurified anti-ELA antibody appeared to perform better at capturing ELA peptides from solution (Fig. 5.9). Hence, further studies were continued using Protein A/G magnetic beads and unpurified antibody.
Figure 4.9. Validation of antibody affinity for the ELA isoforms. Antibody coated protein A/G or Dynabeads M-280 tosylactivated were added to a solution containing ELA-11, ELA-21 and ELA-32 at the indicated concentrations (5 ng/mL, or 250 ng/mL). Immunoprecipitated peptides were analysed on mass spectrometer; chemerin antibody was used as a negative control. Data represent Mean±STD.

4.3.6 Analysis of Immunoprecipitated Human Kidney and Coronary Artery Extracts on Triple Quadrupole Mass Spectrometer

Having shown that the antibody was specific for ELA peptides, peptides were extracted from human kidney, coronary artery, and rat kidney and immunoprecipitated with the antibody before analyses on the TQX-MS. Using this method, no real
evidence of any of the ELA peptides were detected in these samples (Fig. 4.10). Additionally, ELA peptides were also not seen in the rat kidney samples, but as expected, all isoforms of the peptide were present in the positive control. Although ELA-11 had a sharp chromatographic peak, the chromatographic peak of ELA-32 and ELA-21 were very broad and tailed off which became worse in tissues where the levels of these peptides are too low (Fig. 4.10). Hence, to improve the chromatogram of the longer ELA isoforms, different chromatographic columns were tested, including Acquity UPLC BEH C18 and monolith columns. However, none of these showed improvement to ELA-32 and ELA-21 chromatogram (data not shown).
None of the ELA peptides was detected in these samples as shown by the difference in retention times between control and sample chromatograms; Red arrow indicate expected retention time for each peptide. ELA-11, being more hydrophobic had longer retention times than the hydrophilic ELA-32 and ELA-21. MRM, multiple reaction monitoring.
Since the chromatographic profile of ELA-32 and ELA-21 did not improve with the different columns tested, another strategy was to change or modify the mobile phase. Given the strong positive charge on these peptides, an ideal mobile phase will need to ion-pair to reduce the overall positive charge on each peptide. Strong acids, such as trifluoroacetic acid (TFA) are commonly used in the chromatography of charged peptides and proteins as ion-pairing agents (Mysling et al., 2010). This reduces the ability of charged peptides to form electrostatic interactions with the stationary phase by decreasing hydrophilicity and hydrogen bonding potential. Consequently, a better chromatogram is obtained for peptides or proteins but at the expense of better sensitivity due to the inability of the peptides/proteins to re-ionise effectively in the mass spectrometer. TFA was added to the mobile phase to improve the chromatogram of longer ELA isoforms. Using TFA as mobile phase, the tailing off observed on the peak was removed entirely. It was observed that 0.01% TFA showed the best balance between improved sensitivity and chromatogram quality and was selected for subsequent analysis described from now on.

4.3.7 Analysis of Immunoprecipitated Extracts on Orbitrap Mass Spectrometer

Extracted peptides immunoprecipitated with ELA antibody-coated magnetic beads were analysed on the ThermoFisher Orbitrap mass spectrometer using 0.01% TFA as mobile phase. None of the ELA peptides was detected in either a human coronary artery or kidney samples. To verify these results extracts recovered after LC-MS/MS were analysed by ELISA. The concentration of ELA by ELISA was 637.1±151 pg/ml in the kidney and 253.2±168 pg/ml in the coronary artery (Fig. 4.11A). Because the concentration detected in the coronary artery was deemed too low, it was removed from subsequent studies described hereafter.
Moreover, glioblastoma (GBM) was suggested to overexpress ELA (Ganguly et al., 2019). Therefore, ELA concentration in GBM samples was measured by ELISA, which detected a high concentration of the peptide in this tissue, 8013±1041 pg/ml, as well as in normal brain (control) 9410±2139 pg/ml (Fig. 4.11B). Hence, control and GBM brain were added to subsequent experiments described henceforth to increase the chance of detecting endogenous ELA peptides.

**Figure 4.11. Analysis of ELA peptides in human tissues.**

A, The concentration of ELA peptides in kidney and coronary artery samples measured by ELISA after immunoprecipitation and LC-MS/MS analysis. B, The concentration of ELA peptides in control (normal) and glioblastoma brain samples. C, An 8-point ELA standards calibration curve used to determine instrument (mass spectrometer) sensitivity for
each ELA isoform; ELA-11, $r^2=0.997$, ELA-21, $r^2=0.996$, ELA-32, $r^2=0.999$; calibration curve range was 10 pg/ml to 50 ng/ml, but the pg/ml concentrations are clustered at the bottom of the curve shown hence only a few points are visible. Data represent mean±SEM.

Pauli et al., 2014, previously extracted ELA peptides by boiling in water followed by acidification, before the resulting samples were concentrated (pooled together). This method allowed detection of ELA-11 endogenously by LC-MS/MS in their over-expression model, but none of the longer isoforms was detected. Hence, this strategy was also explored in the hope of increasing endogenous ELA concentration and thus enhancing detection by mass spectrometry. Human kidney, control brain and GBM samples were extracted using the above method. In parallel, some samples were also immunoprecipitated as described earlier and a mixture of ELA-11, ELA-21 and ELA-32 in 0.1% BSA (surrogate matrix) at 100 ng/ml used as a positive control. Additionally, an 8-point calibration curve (10 pg/ml – 50 ng/ml) was constructed to determine the lower limit of detection (LOD, sensitivity) and upper limit of detection of each ELA isoform. ELA-11 calibration curve ($r^2 = 0.997$) showed that the LOD for this peptide was 100 pg/ml. However, the LOD for the longer isoforms ELA-21 (calibration curve $r^2 = 0.996$) and ELA-32 (calibration curve $r^2 = 0.999$) was five-fold higher at 500 pg/ml (Fig. 4.11C). Additionally, targeted analysis of the samples for ELA-32 ($m/z$: 586.16, 7$^+$ and 581.88, 7$^+$), ELA-21 ($m/z$: 451.39, 6$^+$) and ELA-11 ($m/z$: 447.55, 3$^+$), was unable to detect any of the longer isoforms, ELA-21 and ELA-32 but low levels of ELA-11 was present in the pooled samples (Fig. 4.12A–D).

Furthermore, none of the peptides was detected using the full scan DDA analysis performed in parallel to the targeted analysis. However, ELA-21 peptide with the loss of CH$_3$SH group (-48 Da) on its methionine residue was consistently observed, which may have resulted from collision-induced fragmentation (MS/MS) of parent ion as previously reported for [Pyr$^1$]apelin-13 (Mesmin et al., 2011). However, this was ~3Da higher than the expected mass of ELA-21.
Figure 4.12. Representative chromatograms of endogenous ELA-11 peptide detected in human GBM tissues. A,C; ELA-11 positive control chromatogram (A) and its carbon-13 isotopes (C); B,D; endogenous ELA-11 chromatogram and its carbon-13 isotopes (D).
Furthermore, the acquired raw LC-MS/MS data files were put through Uniprot database searching as previously described. However, this technique was unable to identify ELA peptides in any of the samples. Several other potentially important proteins and peptides were observed which reflect the peptidome of these tissues. Whilst most of these proteins/peptides were expressed in both control brain, and GBM, including neuromodolin and neurosecretory protein, some of the key neuropeptides were only seen in the control but not the GBM sample. This included Cocaine- and amphetamine-regulated peptide (CART), neuropeptide Y (NPY), cholecystokinin (CCK) and Pro-SAAS family of peptides (Table 4.4). The differentially expressed proteins and peptides found in control and GBM samples were shown in figure 4.13 and figure 4.14.

Table 4.4. Selected essential peptides present in control (normal) brain but not glioblastoma.

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<th>Protein</th>
<th>-10logP</th>
<th>Mass</th>
<th>Length</th>
<th>ppm</th>
<th>m/z</th>
<th>RT</th>
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<td>0.3</td>
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<td>Little SAAS</td>
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<td>2522.30</td>
<td>24</td>
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<td>1385.317</td>
<td>58.72</td>
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<td>2041.94</td>
<td>18</td>
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<td>1.3</td>
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<td>1.5</td>
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<td>0.1</td>
<td>864.160</td>
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-10\log P = \textit{measure of confidence in sequence matching}; \ ppm = \textit{parts per million}; \ m/z = \textit{mass to charge ratio}; \ RT = \textit{retention time}.
Figure 4.13. Protein-protein interaction networks for expressed in control but not GBM brain tissue. The Interaction network was generated using Search Tools for the Retrieval of Interacting Proteins/Genes (STRING) version 11. Each line represents potential protein-protein interaction for proteins that are involved in similar pathways or have related functions. The type of protein interaction and their effects are colour-coded and shown on the bottom panel.
Figure 4.14. Protein-protein interaction network for proteins expressed in GBM but not control brain tissues. The Interaction network was generated using Search Tools for the Retrieval of Interacting Proteins/Genes (STRING) version 11. Each line represents potential protein-protein interaction for proteins that are involved in similar pathways or have related functions. The type of protein interaction and their effects are colour-coded and shown on the bottom panel.
In this study, I have developed an LC-MS/MS method for the detection and quantification of ELA peptides in biological fluids and tissues. Using this method, I was able to identify the \textit{in vitro} generated metabolites of ELA-32 in human plasma and tissue. Several potentially functional shorter fragments were identified, the most important of which is ELA-16 due to its disulphide bridge that will likely make it more resistant to endoproteases. In addition, attempts to use this assay to identify the most abundant endogenous isoform of ELA peptides present in human tissues, was very challenging. However, targeted MS/MS analysis found potential evidence for endogenous ELA-11 in human tissues, but its levels were generally low.

The \textit{in vitro} plasma half-life of ELA-32 observed in this study was approximately 47 minutes. This was consistent with pilot studies where peptide recovery from samples extracted in the absence of a chaotrope was relatively low compared to when guanidine hydrochloride was present in the extraction medium. Considering the robustly charged nature of ELA-32 peptide, a plausible reason for this may be plasma protein binding which could contribute to the reduced proteolytic degradation and increased stability compared to apelin peptides. It should also be noted that the N-terminal Gln residue in this peptide was pyroglutaminated, which may further protect it from N-exoproteases (Cynis et al., 2008). However, a previous \textit{in vitro} degradation study of the same peptide ([\text{Pyr}^1]ELA-32) in rat plasma reported a half-life of $\sim$2 minutes (Murza et al., 2016). A similar discrepancy between \textit{in vitro} plasma half-life in human and rodents were recently reported for [\text{Pyr}^1]apelin-13 (Onorato et al., 2019), thereby highlighting potential differences in the repertoire of proteolytic enzymes in rodents and humans. Additionally, the observed plasma half-life of ELA-32 in this study was significantly longer than that observed with apelin peptides, whose plasma half-life ranged from 2-8 minutes \textit{in vitro} (Japp and Newby, 2008; Japp et al., 2008; Murza et al., 2016). Thus, perhaps one the reasons two ligands activate the same apelin receptor is that ELA may be the most resistant to proteolytic degradation, thereby prolonging the beneficial effects of apelin receptor signalling.
Mature ELA-32 peptide has two potential furin cleavage sites characterised by di-arginine residues (Hosaka et al., 1991). Based on this, the existence of two other isoforms of the ELA peptide, ELA-21 and ELA-11 were proposed (Chng et al., 2013; Pauli et al., 2014). A previous overexpression study identified ELA-11 but not ELA-21 or ELA-32 (Pauli et al., 2014), and more recently in a buffer-based ELA-32 digestion study, ELA-11 was identified as a furin metabolite (Chen et al., 2017). ELA-21, which is one amino acid less than the expected furin cleavage site, has never been detected in digestion studies. Interestingly, apelin-55 also contain these di-arginine residues, and it was shown that PCSK3 cleave immediately after these dibasic residues in the N-terminal to generate apelin-17 and apelin-13 (Shin et al., 2013, 2019). Here, ELA-11 and ELA-22 were identified as fragments of ELA-32 generated in vitro in human plasma, but there was no evidence of ELA-21 either in the plasma or tissue homogenate samples. Consistent with our results, Murza et al., (2016) observed the generation of ELA-22 but not ELA-21 in rat plasma in vitro. Taken together, these studies suggest a potential role of PCSK3 and other furin-like proprotein convertases in the cleavage of ELA-32 generating ELA-22 and ELA-11 but not ELA-21 in human plasma.

Besides the furin, no other enzyme has been proposed to cleave ELA-32. ACE2 is well known for its ability to cleave apelin peptides which have similar chemical properties and C-terminal amino acids to ELA peptides. ACE2 preferentially cleaves immediately after a proline residue in the C-terminal and its activity has been demonstrated for only three peptides namely angiotensin II, des-Arg9-bradykinin and apelin peptides (Vickers et al., 2002; Yang et al., 2017c). Here, cleavage immediately after the terminal proline residue, resulting in the generation of ELA-161-16 (CLQRCMPLHSRVP|F) from des-Pro16-ELA-16 and ELA-191-17 (KHNCLQRCMPLHSRVP|F) from des-Pro19-ELA-19 were observed, which may suggest the activity of ACE2. These potential ACE2 substrates may have been generated from ELA-321-31, which was the most abundant C-terminal metabolite identified. Importantly, these fragments are likely to be G-protein biased ligands of the
apelin receptor, given that the loss of this phenylalanine residue impairs β-arrestin recruitment (Murza et al., 2016). However, it is not clear whether like bradykinin where an initial proteolytic removal of the C-terminal arginine (Arg⁹) residue is a prerequisite for ACE2 activity (Vickers et al., 2002), the removal of C-terminal proline (Pro₃₂) residue is a prerequisite for the observed ACE2 activity.

Furthermore, several fragments of ELA-32 resulting from the loss of N-terminal amino acids were identified, including ELA-20 and ELA-16. Whether or not ELA-20 was generated from ELA-21 due possibly to instability of the latter peptide in plasma is unknown, but further interrogation of the data identified the corresponding 12 amino acid residues enzymatically removed from the N-terminus of ELA-20, suggesting that this was a primary cleavage site. ELA-16 and ELA-19 were generated much earlier (around 2 mins of incubation), and ELA-16 was previously shown to have a similar affinity for the apelin receptor as ELA-32 (Murza et al., 2016). Therefore, ELA-16 fragment may be of potential importance as a tool compound for further studies as it contains a disulphide bridge in its N-terminal, which may stabilise it from proteolytic degradation, but ELA-19 is novel and has never been reported previously.

Previous studies have shown that ELA-32 fragments up to the last 11 amino acids at the C-terminus (ELA-11) were able to bind and activate the apelin receptor (Murza et al., 2016; Yang et al., 2017b), suggesting that these metabolites would have biological activity at the apelin receptor. Moreover, ELA-11 inhibited cAMP release with similar potency as ELA-32 and ELA-21 at the human apelin receptor but was less (~10 fold) able to recruit β-arrestin when compared to the longer isoforms (Murza et al., 2016; Yang et al., 2017b). This may suggest that shorter fragments potently activate beneficial signalling pathways with decreased ability to induce receptor desensitisation, thereby prolonging signalling activity. In support, ELA-14 also retained a subnanomolar affinity for the apelin receptor, reducing arterial pressure and exerting ionotropic effects on the heart both ex vivo and in vivo (Murza et al., 2016). Similarly, recent studies suggest that ELA-11 was comparable to ELA-32 in its ability to prevent DNA damage-induced acute kidney injury (Chen et al., 2017) and suppression of
tumour growth (Soulet et al., 2020). Taken together, these studies suggest that loss of N-terminal amino acids from mature ELA peptide does not adversely affect biological activity both in vitro and in vivo.

It is not known whether shorter fragments like ELA-8 and ELA-9 could bind and activate the apelin receptor, as with apelin, where shorter isoforms could bind (Yang et al., 2017c). A structure-activity relationship study of ELA peptides identified four C-terminal amino acid residues that were critical for receptor binding, namely, His\textsuperscript{26}, Arg\textsuperscript{28}, Pro\textsuperscript{30} and Phe\textsuperscript{31} (Murza et al., 2016). Hence, since ELA-8 and ELA-9 retained these residues, it would be interesting to see whether they are able to bind the apelin receptor or act as antagonists at the receptor. Given that suitable apelin receptor antagonists are lacking, these fragments may present a potential starting pointing for development of ELA-based antagonists for experimental medicine.

The identification of ELA-32\textsubscript{(1-21)}, ELA-32\textsubscript{(1-20)} and ELA-32\textsubscript{(1-19)} may suggest a sequential proteolytic cleavage of ELA-32 from the C-terminal. Interestingly, except ELA-32\textsubscript{(1-20)} and ELA-32\textsubscript{(1-19)} whose corresponding N-terminal metabolite was not found; the corresponding N-terminal metabolites of these fragments were identified. Additionally, ELA-32\textsubscript{(1-12)}, ELA-32\textsubscript{(1-13)}, ELA-32\textsubscript{(1-14)} and ELA-32\textsubscript{(1-16)} resulting from the loss of C-terminal amino acids were identified. Notably, these fragments are unlikely to have biological activity at the apelin receptor since the critical pharmacophore required for binding are present in the C-terminal, which has been removed (Murza et al., 2016). Conversely, for [Pyr\textsuperscript{1}]apelin-13, the RPRL motif critical for apelin receptor binding is present on the N-terminal (Langelaan et al., 2009; Nyimanu et al., 2019a; Read et al., 2019a). Therefore, it appears that these peptides (apelin and ELA) evolved in such a way that any enzymatic degradation preserves their pharmacophore.

The most predominant endogenously generated isoform of ELA in mammals remains unknown. Following its discovery, Chng et al., (2013) reported on the identification of mature ELA-32 in zebrafish using a specific N-terminal antibody. Similarly, after
overexpression of ELA mRNA expression in zebrafish, Pauli et al., 2014 reported on the identification of ELA-11 by mass spectrometry. Conventional immunological methods are unable to distinguish between the various isoforms, hence using mass spectrometry I sought to identify for the first time in vivo the most abundant ELA isoform. Initial studies on human kidney and coronary artery tissues failed to detect any ELA isoform in these tissues. To enrich for ELA peptides in these samples following tissue extraction, ELA peptides were immunoprecipitated before mass spectrometry, but the peptides were still mostly undetectable. However, targeted LC-MS/MS analysis provided potential evidence for endogenous ELA-11, but the levels were low. Owing to the poor chromatographic properties of the longer isoforms, I was unable to detect them endogenously, and it is not clear whether ELA-11 identified was a cleavage product of these longer isoforms. Thus, further studies could build on this work to develop methods for accurate detection and quantification of the longer isoforms as well as ELA-11.

Furthermore, although mass spectrometry could barely detect endogenous ELA-11, ELISA assay detected much higher concentrations of the ELA peptides. For example, the same samples where ELA peptides were not detected by mass spectrometry, the ELISA assay measured ELA concentrations of 637.1±151 and 253.2±168 pg/ml, respectively. The lack of detection on mass spectrometry may partly be explained by decreased sensitivity due to physicochemical properties of the peptide which may have been exacerbated by TFA in the mobile phase (Apffel et al., 1995; García et al., 2002). Although this unusual physicochemical properties is uncommon in most biologically active peptides, a similar property was reported for the neuropeptide salusin-β (Fujimoto et al., 2017). Additionally, the seeming discrepancy between the ELISA and mass spectrometry data observed may be explained by the fact that the antibody used in the ELISA assay has an affinity for several ELA isoforms including ELA-32, ELA-21, ELA-11 and any other potential ELA fragment that might be present in the tissues. Hence, the ELISA measured cumulative signal from all the potential ELA isoforms present while the mass spectrometry measured signal from each
specific isoform. Regardless, this study provided possible evidence for the endogenous production of ELA-11.

In conclusion, we have shown that ELA-32 may be the most stable of the apelin receptor ligands (apelin and ELA) with an *in vitro* half-life of approximately 47 mins in plasma. Additionally, several biologically active metabolites of ELA-32 were identified, notably ELA-16 and ELA-19 or ELA-22. Considering that ELA-16 has a di-sulphide bridge in its N-terminal, it may be the most stable isoform of the peptides that retain activity at the apelin receptor. Attempts to identify the endogenous levels of these peptides proved challenging due to their physicochemical properties which predispose ELA peptides to oxidation and poor chromatographic properties. However, there was potential evidence for the identification of ELA-11. Given the beneficial effects of ELA signalling in various disease states including renal (Chen *et al.*, 2017; Li *et al.*, 2020c; Xu *et al.*, 2020) and cardiovascular diseases (Read *et al.*, 2019a), the long half-life observed here may suggest that ELA peptides could be a potential therapeutic option for managing these conditions.

In terms of further studies, mobile phase selection may be an important consideration for proper chromatographic separation of ELA peptides in any matrix. Conventional mobile phase such as formic acid does not seem appropriate for chromatography of ELA peptides due to the peak tailing observed, making it necessary to consider suitable ion-pairing reagents such as TFA. Additionally, one of the main difficulties with quantification of ELA peptides was its ability to bind unspecifically to proteins and materials. Therefore, prior to immunoprecipitation of the peptide with the appropriate antibody, it may be necessary to perform solid-phase extraction of ELA peptides from the matrix of interest (such as homogenates) in the presence of chaotropes like guanidine hydrochloride. This may minimise the unspecific binding of ELA to other proteins, and the solid-phase extraction step may help remove some of the high abundance proteins which may bind to the antibody or interfere with sensitivity to ELA peptides.
Furthermore, it may be possible that ion suppression from high abundance proteins like haemoglobin and actins contribute to the decreased sensitivity of the LC-MS/MS for ELA peptides. Thus, appropriate methods for removal of high abundance proteins may be interrogated and the best method that is suitable for the extraction of ELA peptides selected. In this study, acetonitrile was used to precipitate peptides from homogenates and plasma samples before followed by solid-phase extraction before mass spectrometry. However, I did not have enough time to investigate the potential utility of this approach in the preparation of samples for endogenous ELA detection. So, this could be a starting point of further efforts to characterise ELA peptides by mass spectrometry fully. Another method previously used in the literature is the immunoaffinity chromatography using multiple affinity removal systems (MARS) capable of depleting 7 or 14 abundant proteins, which was shown to increased detection of low abundant proteins by an average of 4-fold (Haudenschild et al., 2014; Tu et al., 2010).
Chapter 5 Development and Validation of an LC-MS/MS Assay for Detection and Quantification of In Vivo-Derived Metabolites of [Pyr1]Apelin-13 in Humans

5.1 Introduction

Apelin is an endogenous ligand of the apelin receptor, initially characterised from bovine stomach extracts as a 77-amino acid preproprotein (Tatemoto et al., 1998). The prepro-apelin is further cleaved into shorter but functional fragments including apelin-36, apelin-17, apelin-13 and [Pyr1]apelin-13 that contain an evolutionary conserved 12-amino acid C-terminal (Habata et al., 1999; De Mota et al., 2000; Tatemoto et al., 1998). [Pyr1]apelin-13 was subsequently identified as the most predominant isoform of the apelin family of peptides in the cardiovascular system (Chen et al., 2003; Maguire et al., 2009), and the primary circulating form of the peptide (Zhen et al., 2013).

In the cardiovascular system, apelin is a potent endogenous inotropic agent (Maguire et al., 2009); decreased blood pressure when infused into rats and dilating resistance vessels when infused into human forearm (Japp et al., 2008; El Messari et al., 2004; Read et al., 2019b). Apelin also causes nitric oxide-dependent (Salcedo et al., 2007), and prostanoid-dependent (Maguire et al., 2009), vasodilation of human splanchnic artery. In endothelium-denuded vessels, apelin acts a vasoconstrictor via a direct action on vascular smooth muscle cells (Maguire et al., 2009; Pitkin et al., 2010). Based on these beneficial effects, apelin was proposed as a potential therapeutic target in cardiovascular diseases. For example, apelin administration protected mice from heart damage in a heart failure model (Sato et al., 2013), and ameliorated the development of pulmonary arterial hypertension in rats (Read et al., 2019a; Yang et al., 2019) and humans (Brash et al., 2018). In addition, the protective effects of apelin
have been reported in metabolic diseases where it decreased adiposity, serum insulin and increased insulin sensitivity (Galon-Tilleman et al., 2017; Nyimanu et al., 2019a); and in renal diseases where it decreased acute renal injury and fibrosis (Marsault et al., 2019). However, these beneficial effects of apelin peptides are limited by the rapid in vivo metabolism, suggesting that to harness its potential therapeutic effect fully, apelin peptides should be made resistant to proteolysis.

Previous studies investigating the metabolism of apelin peptides were primarily conducted in plasma in vitro or in rodent models, neither of which may represent metabolism in humans (Onorato et al., 2019). These studies demonstrated that apelin peptides are very labile in plasma with a half-life less than 1-5 minutes in vitro (Murza et al., 2014, 2016; Vickers et al., 2002; Wang et al., 2016; Yang et al., 2017c). This plasma instability has to date been attributed the enzymatic activity of neprilysin (McKinnie et al., 2016) and angiotensin-converting enzyme II (ACE2) (Murza et al., 2014; Wang et al., 2016; Yang et al., 2017c), and more recently plasma kallikrein (Fischer et al., 2019; Wang et al., 2019). Therefore, understanding the degradation profile of [Pyr¹]apelin-13 in vivo in humans would be very useful in attempts to develop stable analogues of the peptide as tool compounds or for preclinical development as a potential cardiovascular therapeutic.

5.1.1 Hypotheses and Aims

To date, no studies have investigated the metabolism of apelin peptides in vivo in humans. Interestingly, ACE2 was suggested to hydrolyse [Pyr¹]apelin-13 to [Pyr¹]apelin-12(1-12) in vitro (Yang et al., 2017c). Therefore, it was hypothesised that [Pyr¹]apelin-12(1-12) was the principal metabolite of [Pyr¹]apelin-13 in humans. The aim of this study was:

1. To develop a highly sensitive mass spectrometry-based method for detection and quantification of apelin peptides in plasma.
2. Use this method to measure intact [Pyr¹]apelin-13 and its metabolites generated \textit{in vivo} in humans, following a constant 120 minutes infusion of the peptide.
5.2 Materials and Methods

5.2.1 Cell Signalling Assays

5.2.1.1 β-Arrestin Assay

[Pyr<sup>1</sup>]apelin-13 infused into healthy volunteers were first assessed for the ability to bind the apelin receptor and recruit β-arrestin in CHO cells artificially expressing the receptor (n = 4 independent experiments). The concentration of [Pyr<sup>1</sup>]apelin-13 used in these experiments was (30 µM-10 pM). Experiments were performed as described in section 2.8.2 in duplicates in at least 4 independent experiments.

5.2.1.2 cAMP Assay

[Pyr<sup>1</sup>]apelin-13 (1 pM-0.3 µM) was also assessed for the ability to inhibit forskolin-induced cAMP accumulation (n=2 independent experiments) in CHO cells artificially (stably) expressing the apelin receptor as described in section 2.8.1.

5.2.2 Left Ventricular Catheterisation and Chronic Infusion of [Pyr<sup>1</sup>]Apelin-13 in Rats

To further assess the pharmacology of [Pyr<sup>1</sup>]apelin-13 peptide before infusion in humans, male Sprague-Dawley rats (saline, n=8; [Pyr<sup>1</sup>]apelin-13, n=22) were used. These rats underwent jugular vein cannulation for infusion of test peptides and catheterisation as described in Section 2.14. They were then infused either with [Pyr<sup>1</sup>]apelin-13 (50 nmol, 0.5 ml) or saline (0.5 ml).

5.2.3 Study Protocol

The clinical study protocol was shown in chapter 2, section 2.12.1. Six healthy volunteers (3 male and 3 female, mean age 43.8±6.9, with body mass index within the normal range of 23.0±1.0) for infusion were infused with [Pyr<sup>1</sup>]apelin-13 (135 nmol/min) at a constant rate of 1 ml/min for 120 mins. Venous blood samples were
collected from the contralateral arm before and after infusion into EDTA tubes and centrifuged to collect plasma. Additional control samples were obtained from 6 donors (3 male and 3 female) within a similar age group who did not receive the apelin infusion for comparison.

5.2.4 Optimisation of Extraction Methods for Plasma [Pyr1]Apelin-13

To identify the best method for extraction of [Pyr1]apelin-13 human plasma, four different conditions were tested (section 2.12.2). These included protein precipitation with 300 μl of 75%, 80%, 85% or 90% ACN in water (v/v) (condition 1) or the same ACN solution with 0.1% formic acid (FA) (v/v) (condition 2); condition 3 where guanidine hydrochloride (6 M, 25 μl) was mixed with 50 μl plasma before protein precipitation 300 μl 80% ACN in water with or without 0.1% FA (v/v); and condition 4, where 450 μl deionised water or 50 mM ammonium bicarbonate (pH 8) or 1% FA (v/v) was mixed 50μl plasma. Following solid-phase extraction, samples were evaporated reconstituted in 150 μl 0.1% FA in water, and 15 μl was injected onto an LC-MS/MS system.

5.2.5 [Pyr1]Apelin-13 LC-MS/MS and SRM Based Detection Method Development

LC-MS/MS instrumentation used for the quantitation of [Pyr1]apelin-13 included an H-Class Acquity (Waters) attached to a triple quadrupole mass spectrometer (Waters), and details of the analytical conditions were described in section 2.12.3. Full scan analysis of [Pyr1]apelin-13 showed that the most predominant ion in the spectrum was [M+4H]4+ charge state, so this was used for fragmentation. Product ion spectrums were collected over a range of 100 to 1600 m/z and two ions used for selected reaction monitoring (SRM) optimisation (m/z 424.6 and 408.55). The 424.6 ion corresponded to the b11 fragment and the 408.55 ion was derived from the loss of a methyl-sulphide group from the methionine on the b11 ion, as previously described (Mesmin et al.,
Optimal conditions for the two SRM transitions for [Pyr\(^1\)]apelin-13 were 384.2/408.55, 384.2/424.6 with collision energy values of 14 and 12 eV respectively. The internal standard used the same collision energy but targeted transitions of 387.45/412.88 and 387.45/428.26. Peptide peak areas were integrated using the TargetLynx program associated with Masslynx V 4.2 (Waters), and peak area ratios were generated against the corresponding stable isotope-labelled internal standard peptide peak.

### 5.2.6 Precision and Accuracy of the Extraction Method

For quantification of [Pyr\(^1\)]apelin-13 in human plasma samples, an 8-point calibration line, initially 25 pg/ml to 50 ng/ml (sensitivity study) and then 1 ng/ml to 1000 ng/ml (for quantitation) was generated in duplicate. In addition, four QCs (1, 3, 100 and 800 ng/ml) were together with [Pyr\(^1\)]apelin-13 internal standard (25 ng/ml) were generated and extracted six times to assess precision and accuracy of the method as described in Section 2.12.5. The percentage relative error was calculated by subtracting the expected concentration from the measured concentration divided by expected concentration and multiplying the result by 100%. Percentage coefficient of variation (imprecision of the assay) was determined by dividing the standard deviation by the mean and multiplying 100%.

### 5.2.7 Peptide Identification using High-Resolution Mass Spectrometry

Samples were analysed on mass spectrometry as described in Section 2.12.6 to identify the potential metabolites generated *in vivo*. Raw data files were searched through Peaks Studio software as defined in section 2.13 to identify apelin peptides. In addition, the LC-MS/MS data were interrogated for potential [Pyr\(^1\)]apelin-13 metabolites by searching for all potential cleavage products from the parent peptide in the RAW data files using the Qualbrowser software package (Thermofisher). The \( m/z \)
values for these peptides at multiple charge states are displayed in Supplementary Table 5.1.

**Table 5.1. List of theoretical m/z values of [Pyr]\(^1\)apelin-13 metabolites with charge states up to 4+**

<table>
<thead>
<tr>
<th>Apelin peptides</th>
<th>C-terminal metabolites</th>
<th>N-terminal metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M+1H</td>
<td>2+</td>
</tr>
<tr>
<td>1-13</td>
<td>QRPRLSHKGPMP</td>
<td>1533.8107</td>
</tr>
<tr>
<td>1-12</td>
<td>QRPRLSHKGPMP</td>
<td>1386.7423</td>
</tr>
<tr>
<td>1-11</td>
<td>QRPRLSHKGP</td>
<td>1289.6895</td>
</tr>
<tr>
<td>1-10</td>
<td>QRPRLSHKG</td>
<td>1158.6491</td>
</tr>
<tr>
<td>1-9</td>
<td>QRPRLSHG</td>
<td>1061.5963</td>
</tr>
<tr>
<td>1-8</td>
<td>QRPRLSHK</td>
<td>1004.5746</td>
</tr>
<tr>
<td>1-7</td>
<td>QRPRLSH</td>
<td>876.4799</td>
</tr>
<tr>
<td>1-6</td>
<td>QRPRLS</td>
<td>739.4209</td>
</tr>
<tr>
<td>1-5</td>
<td>QRPRL</td>
<td>652.3889</td>
</tr>
<tr>
<td>1-4</td>
<td>QRPRL</td>
<td>539.3049</td>
</tr>
<tr>
<td>1-2</td>
<td>QRPRLS</td>
<td>286.1510</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Most abundant</th>
<th>Least abundant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apelin peptides</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Where more than one precursor ion was identified, the abundance of the two ions was assessed and compared. Retention times of identified peptides were included.

### 5.2.8 Statistical Analysis

Peak areas were expressed as mean±SD while data from in vivo studies were described as mean±SEM. Data were first tested for normality using the D’Agostino test and were normally distributed, Student’s *t*-test was performed to test for differences. A *p* value of < 0.05 was considered statistically significant.
5.3 Results

5.3.1 Validation of Biological Activity of [Pyr^1]Apelin-13 Peptide

Since the [Pyr^1]apelin-13 used in this study were synthesised and more stringently purified to satisfy the ethical requirement for human infusion, the biological activity of the peptide was verified in cell-based assays and in vivo in rats. In cell-based assays, [Pyr^1]apelin-13 potently (pD\textsubscript{2} (negative log\textsubscript{10} EC\textsubscript{50}) = 8.50±0.05) recruited β-arrestin following binding to the apelin receptor (Fig. 5.1A). Similarly, in cAMP assays which measures the G protein activity of the apelin pathway, [Pyr^1]apelin-13 potently inhibited forskolin-induced cAMP release with pD\textsubscript{2} of 9.82±0.26 (Fig. 5.1B).

![Figure 5.1](image)

*Figure 5.1. The in vitro activity of [Pyr^1]apelin-13 in cell-based β-arrestin (A) and cAMP (B) assays. EC\textsubscript{50} = the concentration of agonist producing 50% of the maximum response to that agonist. Data represents mean±SEM.*

To evaluate the physiological effects of the peptide, [Pyr^1], apelin-13 was administered intravenously to anaesthetised rats and the effect on cardiovascular parameters assessed. In vivo, [Pyr^1]apelin-13 significantly decreased systolic pressure (Fig.5.2A, -15.15±1.6 vs 3.27±1.7 mmHg, p<0.001). The effect of [Pyr^1]apelin-13 on cardiac output and heart rate were also examined. [Pyr^1]apelin-13 significantly increased
cardiac output (1575±202.8 vs 377.4±144 RVU/min, \(p<0.005\)) and heart rate (6.65±1.6 vs -0.62±1.9 bpm, \(p<0.05\)) when compared to saline controls (Fig. 5.2B, C).

![Figure 5.2. The in vivo activity of [Pyr\(^1\)]apelin-13 in anaesthetised rats. A, systolic pressure; B, cardiac output (CO); C, heart rate (HR); bpm= beats per minute, RVU/min = relative value units/minutes; mmHg = millimetre mercury.](image)

### 5.3.2 Validation of Extraction Method for Extraction of Apelin Peptides from Human Plasma

The method for extracting apelin peptides from plasma was carefully evaluated by spiking [Pyr\(^1\)]apelin-13 into plasma and monitoring its recovery using different extraction methods. It was observed that 80% ACN in water (% v/v), 75% ACN in 0.1% FA (% v/v), 75% ACN in 0.1% FA (% v/v) followed by SPE, 80% ACN in water or in 0.1% FA (v/v) plus GuHCl gave 82.0%, 99.5%, 97.7%, 95.0% and 82.0% recovery respectively (Fig. 5.3). The 80% ACN in water plus GuHCl followed by SPE resulted in slightly lower recovery than the 75% ACN with 0.1% FA; however, this condition was chosen for further work for two reasons. The addition of an SPE step results in cleaner extracts prior to LC-MS/MS analysis, and the addition of the GuHCl (a potent chaotrope) will disrupt peptides and potential metabolites from binding to albumin and other proteins in plasma, increasing their recovery during the precipitation phase.
Figure 5.3. Recovery of [Pyr$^1$]apelin-13 from plasma using different extraction methods. Protein precipitation with 80% ACN in water in the presence of half-volume guanidine hydrochloride (GuHCl) followed by solid-phase extraction was used for subsequent experiments. Data represent mean±SD.

### 5.3.3 The Sensitivity of the Extraction and Quantification Method

To assess the lower limit of detection (LOD, sensitivity) of the quantification method, an initial 8-point calibration curve ranging from 25 pg/ml to 50ng/ml using ELA-21 as internal standard was generated. The two SRM transitions (384.2 > 408.55 and 384.2 > 424.6) of [Pyr$^1$]apelin-13 described above were selected from the precursor peptide ion m/z 384.2 to increase sensitivity and selectivity of the analysis (Kuhn and Carr, 2016). The Assay was linear over the range of 25 pg/ml-50 ng/ml and a correlation coefficient ($r^2$) of 0.96 (Fig. 5.4). This confirmed that the extraction and quantification method was sensitive and able to detect [Pyr$^1$]apelin-13. However, ELA-21 did not work well as the internal standard. Therefore, assay imprecision, that is, percentage
coefficient of variation (%CV) was not determined, but a new stable isotope labelled [Pyr¹]apelin-13 was obtained and used as an internal standard for all subsequently apelin analysis. Also, the concentration of [Pyr¹]apelin-13 detected in some samples from infused patients were above the top of the calibration curve, 50 ng/ml and therefore deemed inadequate for accurate quantification. Hence, a broader range calibration line was used as described below.

Figure 5.4. Assessment of the sensitivity of LC-MS Method for quantification of [Pyr¹]apelin-13 in human plasma.
5.3.4 Endogenous Levels of Apelin in Human Plasma

Interestingly, in both unspiked blank (without endogenous peptide digestion) and standard 1 (spiked with [Pyr₁]apelin-13 at 25 pg/ml), a strong peak with a retention time of 3.66±1 mins corresponding to [Pyr₁]apelin-13 was observed (Fig. 5.5A, B). Other peaks were also observed in each transition with a retention time of 3.44±1 mins which may represent oxidised [Pyr₁]apelin-13 (Fig. 5.5A-D).

![Figure 5.5. Representative chromatograms of [Pyr₁]apelin-13 in human plasma. A-B, two MS transitions (384.2 > 408.55, and 384.2 > 424.6) of [Pyr₁]apelin-13 in unspiked human plasma. [Pyr₁]apelin-13 peak indicated by a black arrow. C-D, two MS transitions of [Pyr₁]apelin-13 in standard 1 spiked with 25 pg/ml of [Pyr₁]apelin-13.](image-url)
Consequently, all subsequent blank samples were first incubated at 37 °C for at least 2 hrs to degrade any endogenous apelin peptides that might be present (See fig. 5.5). Additionally, plasma from six control donors who did not receive [Pyr¹]apelin-13 infusion was analysed to verify the endogenous apelin observed. As shown in figure 5.6, standard 1 (STD1) where [Pyr¹]apelin-13 was spiked at 1 ng/ml showed a strong chromatographic peak with retention time, 3.66 mins. Also, this chromatographic peak was present in the donor samples, suggesting that endogenous [Pyr¹]apelin-13 was detectable in these samples (Fig. 5.6).
Figure 5.6. Representative chromatograph obtained from the six donor controls that were not infused with [Pyr<sup>1</sup>]apelin-13. STD1, standard 1 (1ng/ml [Pyr<sup>1</sup>]apelin-13); D1-6, donors 1-6.
5.3.5 Precision and Accuracy of the Extraction and Quantification Method

In order to more accurately quantify [Pyr\(^1\)]apelin-13 and assess the precision and accuracy of the assay, a stable isoform labelled [Pyr\(^1\)]apelin-13 was used as internal standard, together with four QCs extracted six different times. Another 8-point calibration line range (1-1000ng/ml) was prepared using [Pyr\(^1\)]apelin-13 and extracted together with the internal standard. A linear calibration line was observed over this range with a correlation coefficient \((r^2) = 0.99\) (Fig. 5.7).

![ Calibration curve of [Pyr\(^1\)]apelin-13 in human plasma. ](image)

For quantification, the lower limit of quantification (LLOQ) of [Pyr\(^1\)]apelin-13 used was 1 ng/ml. The percentage relative error (% RE) was evaluated for all calibration standards and shown to be less than 20% at the LLOQ and below 15% at other levels, which conformed with the bioanalytical method validation guidelines (DeSilva et al., 2003). The precision and accuracy of the QC samples showed that the method was robust and accurate. The LLOQ samples returned a %CV of 8.0 and %RE of 15.5, whilst the other QC levels had %CVs below 6.1 and %RE's below 8.4 (Table 5.2).
representative chromatogram obtained from calibration standards 1 and 8 are shown in Figure 5.8.

Table 5.2. [Pyr\textsuperscript{1}]apelin-13 calibration curve statistics.

<table>
<thead>
<tr>
<th>STD</th>
<th>Concentration (ng/mL)</th>
<th>1</th>
<th>2</th>
<th>Mean</th>
<th>1</th>
<th>2</th>
<th>%RE</th>
<th>%CV</th>
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<tr>
<td>3</td>
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<td>5.23</td>
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</tr>
<tr>
<td>4</td>
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<td>9.97</td>
<td>9.87</td>
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<td>-0.3</td>
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<tr>
<td>5</td>
<td>50.0</td>
<td>53.2</td>
<td>55.1</td>
<td>54.2</td>
<td>6.4</td>
<td>10.3</td>
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</table>

STD = standard, %RE = percentage relative error, %CV = percentage coefficient of variation.
Figure 5.8. Representative chromatogram of calibration standards. LLOQ standard (A,B) shows peaks corresponding to [Pyr^1]apelin-13 at 1 ng/ml (A) and [Pyr^1]apelin-13 internal standard at 25 ng/ml. The upper limit of quantification (C, D), shows peaks corresponding to [Pyr^1]apelin-13.
5.3.6 Plasma Concentrations of [Pyr\(^1\)]Apelin-13 in Healthy Human Volunteer Samples Infused with the Peptide

In samples obtained before infusion of [Pyr\(^1\)]apelin-13, a chromatographic peak for the peptide was undetectable (Fig. 5.9A, B). But samples acquired at the end of the infusion (t=120 minutes) showed strong peaks at 3.68 minutes, corresponding to [Pyr\(^1\)]apelin-13 (Fig. 5.9C, D). The measured concentration of [Pyr\(^1\)]apelin-13 in these samples after 120 minutes was 58.3±10.5 ng/ml.

**Figure 5.9.** Representative chromatogram for [Pyr\(^1\)]apelin-13 and its internal standard in volunteer samples. A-B, chromatograms for samples obtained at t=0 minutes; C-D, chromatograms for samples obtained at t=120 minutes. A, no [Pyr\(^1\)]apelin-13 was
detected; B, [Pyr1]apelin-13 internal standard chromatogram showing 3.67 minutes retention time; C, [Pyr1]apelin-13 chromatogram showing 3.68 minutes retention time; D, [Pyr1]apelin-13 internal standard

5.3.7 Metabolite Identification using High Flow LC-MS/MS and PEAKs Studio

In order to identify potential metabolites of [Pyr1]apelin-13 generated during the 120 minutes infusion period, samples were re-analysed using a high-resolution mass spectrometer. Full scan LC-MS data was interrogated for potential [Pyr1]apelin-13 derived metabolites by comparing extracted ion chromatograms for each analyte in the 0 and 120 minute time points in the Qualbrowser programme. The resulting raw data files were put through a database search using PEAKs Studio software. Peptides that were identified in the 120-minute samples were mainly from the loss of C-terminal region amino acids (Fig. 5.10A, B) and their relative abundance in the samples are displayed in Figure 4.10C. Notably, the most abundant fragments were [Pyr1]apelin-13(1-12), [Pyr1]apelin-13(1-10) and [Pyr1]apelin-13(1-6) but [Pyr1]apelin-13(1-8), [Pyr1]apelin-13(1-7) and [Pyr1]apelin-13(1-5) were also identified through manual database searching (Fig. 5.10B,C). The chromatographic spectra corresponding to each of these metabolites are shown in Fig 5.11A-G.
Figure 5.10. Identification of C-terminal [Pyr1]apelin-13 metabolites in human plasma. A, PEAKS studio extract of apelin sequence showing database-matched sequences. B, the full sequence of the identified metabolites (* indicate the RPRL motif required for receptor binding). C, the relative peak area of the identified metabolites (n=6, data represent mean ±SD).
Figure 5.11. Representative chromatograms of [Pyr1]apelin-13 metabolites identified in 120 minutes samples on Orbitrap Mass spectrometer (nano LC-MS/MS mode). A, [Pyr1]apelin-13 with 8.80 minutes retention time; B, [Pyr1]apelin-13(1-12) with 6.26/6.48 minutes retention time; C, [Pyr1]apelin-13(1-10) with 4.29 minutes retention time; D, [Pyr1]apelin-13(1-8) with 3.25 minutes retention time; E, [Pyr1]apelin-13(1-7) with 4.22 minutes retention time; F, [Pyr1]apelin-13(1-6) with 5.09 retention time; G, [Pyr1]apelin-13(1-5) with 5.88 minutes retention time.
In addition, oxidation of the methionine residue in \([\text{Pyr}^1]\text{apelin-13}\) was identified, however since this modification was also seen in the extracted standards, it could not be ascertained if they occurred \textit{in vivo} or as an artefact of the extraction process (Fig. 5.10A). In addition to \([\text{Pyr}^1]\text{apelin-13}_{(1-12)}\), the most abundant metabolite identified, two other metabolites were present at a sufficient level to generate suitable product ion spectra allowing experimentally acquired fragments to be matched against theoretical ones from the peptide sequence. The two metabolites that this was performed on are \([\text{Pyr}^1]\text{apelin-13}_{(1-10)}\) (Fig. 5.12) and \([\text{Pyr}^1]\text{apelin-13}_{(1-6)}\) (Fig. 5.13).

**Figure 5.12.** Product ion mass spectrum of \([\text{Pyr}^1]\text{apelin-13}_{(1-10)}\). The main ions identified are shown in brackets. \(y\) ions are fragment ions containing the C-terminus, \(b\) ions are fragment ions containing the N-terminus. \(a\) ions are peptide ions containing the N-terminus, formed upon dissociation of a peptide ion at the peptide backbone (that is by the breaking of peptide bonds between amino acid residues). \(b_2, b_5, b_6, b_7, b_8, \& b_9\) represents fragment ions containing the N-terminal amino acids with the subscript number representing the number of amino acids from the N-terminus producing the signal. \(-\text{NH}_3\) represent ions produced by the loss of ammonia molecule.
Figure 5.13. Product ion mass spectrum of [Pyr<sup>1</sup>]apelin-13(1-6). The main ions identified are shown in brackets. y ions are fragment ions containing the C-terminus, b ions are fragment ions containing the N-terminus. a ions are peptide ions containing the N-terminus, formed upon dissociation of a peptide ion at the peptide backbone (that is by the dissociation of C-C bond between amino acid residues). b<sub>2</sub>, b<sub>5</sub>, b<sub>6</sub>, b<sub>7</sub>, b<sub>8</sub> & b<sub>9</sub> represents fragment ions containing the N-terminal amino acids with the subscript number representing the number of amino acids from the N-terminus producing the signal. –NH<sub>3</sub> and –H<sub>2</sub>O represent ions produced by the loss of ammonia and water molecule.

5.3.8 Identification of N-Terminal Metabolites of [Pyr<sup>1</sup>]Apelin-13

Using the same approach described above, several N-terminal metabolites of [Pyr<sup>1</sup>]apelin-13 were identified (Fig. 5.14). Of note, the peak areas of these fragments were lower compared to those observed for the C-terminal fragments. The most abundant N-terminal fragments observed were [Pyr<sup>1</sup>]apelin-13(6-13), [Pyr<sup>1</sup>]apelin-13(11-13), [Pyr<sup>1</sup>]apelin-13(7-13) and [Pyr<sup>1</sup>]apelin-13(10-13) (Fig. 5.14A,B). Other fragments present but low in abundance include [Pyr<sup>1</sup>]apelin-13(3-13), [Pyr<sup>1</sup>]apelin-13(4-13) and
[Pyr$^1$]apelin-13$_{8-13}$. The mass accuracy of the experimentally acquired monoisotopic $m/z$ for these metabolites is shown in Fig. 5.15, and were all within 0.9 ppm of expected values.

![Diagram of N-terminal metabolites of [Pyr$^1$]apelin-13 identified from human plasma. A, the sequence of [Pyr$^1$]apelin-13 N-terminal fragments identified, B, the relative abundance of N-terminal metabolites. * indicate on the RPRL motif of [Pyr$^1$]apelin-13 (A) indicate amino acid residues required for receptor binding; n=6.]

Figure 5.14. N-terminal metabolites of [Pyr$^1$]apelin-13 identified from human plasma. A, the sequence of [Pyr$^1$]apelin-13 N-terminal fragments identified, B, the relative abundance of N-terminal metabolites. * indicate on the RPRL motif of [Pyr$^1$]apelin-13 (A) indicate amino acid residues required for receptor binding; n=6.
Figure 5.15. Representative chromatogram of the N-terminal metabolites of [Pyr$^1$]apelin-13 identified in human plasma. A, [Pyr$^1$]apelin-13$_{(6-13)}$ with retention time of 8.01 minutes, B, [Pyr$^1$]apelin-13$_{(7-13)}$ with retention time of 8.08 minutes, C, [Pyr$^1$]apelin-13$_{(8-13)}$ with retention time of 9.35 minutes, D, [Pyr$^1$]apelin-13$_{(10-13)}$ with retention time of 10.45 minutes, E, [Pyr$^1$]apelin-13$_{(11-13)}$ with retention time of 9.32 minutes. The mass accuracy of the experimentally acquired monoisotopic peak was calculated for each potential metabolite and is included along with the $^{13}$C isotopic cluster for each peptide with their corresponding chromatogram.
## 5.4 Discussion

The apelin system has evolved as a critical therapeutic target in several diseases, including cardiovascular, metabolic and renal diseases. However, the utility of apelin peptides as tool compounds for preclinical experiments is severely hampered by their poor plasma stability. Several studies have investigated the degradation of apelin \textit{in vitro} in rodent and human plasma in the hope of identifying the cleavage sites that could be modified to prolong its half-life, with only very few studies examining the metabolites produced \textit{in vivo} in animals (Murza et al., 2014; Onorato et al., 2019). However, to date, no one has previously investigated the degradation of apelin peptides following infusion in healthy human volunteers.

This study reports the development and validation of a high-resolution LC-MS/MS method for detection and quantification of [Pyr\textsuperscript{1}]apelin-13 and relative quantification of its metabolites \textit{in vivo} in man. It was also showed that this method was robust, reproducible and had a high sensitivity for [Pyr\textsuperscript{1}]apelin-13 with an LLOQ of 1 ng/ml (25 pg/ml sensitivity); and demonstrated the ability to quantify the intact peptide after steady-state infusion for 120 minutes. For the first time in humans (\textit{in vivo}), this study showed that [Pyr\textsuperscript{1}]apelin-13 was cleaved from both the N- and C-termini with the C-terminus being the most susceptible to proteolytic activity. The most abundant metabolite identified was [Pyr\textsuperscript{1}]apelin-13\textsubscript{(1-12)}, which has been reported as the \textit{in vitro} cleavage product of [Pyr\textsuperscript{1}]apelin-13 (Murza et al., 2014; Wang et al., 2016). Additional highly abundant metabolites that were present include [Pyr\textsuperscript{1}]apelin-13\textsubscript{(1-10)} and [Pyr\textsuperscript{1}]apelin-13\textsubscript{(1-6)}, the sequences of which were confirmed using tandem mass spectrometry and manual product ion matching.

The discovery that [Pyr\textsuperscript{1}]apelin-13 was cleaved from both ends was unexpected since to date, only C-terminal metabolites have been described (Wang et al., 2016; Yang et al., 2017c). These findings may, therefore, better explain the volatile nature of apelin peptides in plasma (Mesmin \textit{et al.}, 2010; Zhen \textit{et al.}, 2013; Onorato \textit{et al.}, 2019). It is
worth noting that the C-terminus was more susceptible to proteolytic activity than the N-terminus, whose metabolites were present at approximately 20-fold lower. This may partly be explained by the pyroglutamylation of the N-terminus, which may protect this region from enzymatic activity to some degree. The N-terminus of [Pyr<sup>1</sup>]apelin-13 also contain the RPRL motif critical for ligand binding to the apelin receptor (Langelaan et al., 2009); hence any cleavage is likely to profoundly affect the affinity of these N-terminal fragments for the receptor. A previous study showed in vitro that neprilysin cleaves [Pyr<sup>1</sup>]apelin-13 between Arg<sup>4</sup> and Leu<sup>5</sup> and between Leu<sup>5</sup> and Ser<sup>6</sup> amino acids (McKinnie et al., 2016), thereby making neprilysin the first enzyme identified to date that completely inactivates the peptide. Importantly, this study has now demonstrated the presence of these proposed neprilysin cleavage products, [Pyr<sup>1</sup>]apelin-13<sub>(4-13)</sub> and [Pyr<sup>1</sup>]apelin-13<sub>(6-13)</sub>, in humans in vivo with additional evidence for cleavage of the scissile bond between Leu<sup>5</sup> and Ser<sup>6</sup> given by the detection of [Pyr<sup>1</sup>]apelin-13<sub>(1-5)</sub>. This may suggest that in vivo in humans, neprilysin cleaves and inactivates [Pyr<sup>1</sup>]apelin-13. To date very few studies have investigated metabolism of peptides in vivo in humans. Interestingly, like [Pyr<sup>1</sup>]apelin-13, arginine vasopressin (AVP) was also cleaved from both the C- and N-termini, with carboxypeptidases and post-proline cleaving enzymes cleaving the C-terminus of the arginine vasopressin, while aminopeptidases cleaved the N-terminal region (Carone et al., 1987; Argent et al., 1991; Miao et al., 2008). In contrast, other in vivo studies of this nature identified only single terminus cleavage of Peptide YY (Toräng et al., 2016), growth hormone-releasing hormone (Frohman et al., 1986), liraglutide, a glucagon-like peptide-1 (GLP-1) analogue (Malm-Erjefalt et al., 2010), and big endothelin-1 (Hemsén et al., 1995).

Previous in vitro studies in plasma, suggested that [Pyr<sup>1</sup>]apelin-13<sub>(1-12)</sub> was a metabolite of [Pyr<sup>1</sup>]apelin-13 produced by the enzymatic activity of ACE2 resulting in the removal of the C-terminal phenylalanine (Vickers et al., 2002; Murza et al., 2014; Wang et al., 2016; Yang et al., 2017). However, it was unclear whether this metabolite retained biological activity at the apelin receptor. One study argued that [Pyr<sup>1</sup>]apelin-13<sub>(1-12)</sub> had reduced biological activity compared to the native [Pyr<sup>1</sup>]apelin-13 as
measured by its hypotensive effects in mice (Wang et al., 2016). However, Yang et al. (2017), demonstrated that the ACE2 metabolite, [Pyr]apelin-13\(_{1-12}\) contracted human saphenous vein with sub-nanomolar potencies and was a potent positive inotrope in paced mouse and human heart \textit{ex vivo}. The authors added that [Pyr]apelin-13\(_{1-12}\) was present endogenously in the endothelium of human heart and lungs, and went on to demonstrate that it was biologically active \textit{in vivo} in humans and rodents (Yang et al., 2017c). Similarly, a previous study reported that [Pyr]apelin-13 was cleaved to [Pyr]apelin-13\(_{1-12}\) \textit{in vivo} in rats (Murza et al., 2014). Consistent with these studies, this work now provides clear evidence that [Pyr]apelin-13\(_{1-12}\) is produced endogenously in human plasma \textit{in vivo}, possibly via the activity of ACE2.

[Pyr]apelin-13 was also cleaved between Pro\(^{10}\) and Met\(^{11}\) and between Ser\(^{6}\) and His\(^{7}\) resulting in [Pyr]apelin-13\(_{1-10}\) and [Pyr]apelin-13\(_{1-6}\), but the enzyme-producing these metabolites remain unknown. The corresponding N-terminal fragments of these C-terminal metabolites [Pyr]apelin-13\(_{11-13}\), [Pyr]apelin-13\(_{7-13}\), were also identified. These data were consistent with a previous \textit{in vivo} study in male rats which also identified the C-terminal metabolites (Murza et al., 2014). The authors reported on the accumulation of [Pyr]apelin-13\(_{1-10}\) and [Pyr]apelin-13\(_{1-6}\) signals with time as [Pyr]apelin-13 and [Pyr]apelin-12 levels decreased, suggesting that following ACE2 cleavage of [Pyr]apelin-13 to [Pyr]apelin-12, other unidentified enzymes subsequently cleave both [Pyr]apelin-13 and [Pyr]apelin-12 into [Pyr]apelin-13\(_{1-10}\) and [Pyr]apelin-13\(_{1-6}\). These metabolites, [Pyr]apelin-13\(_{1-10}\) and [Pyr]apelin-13\(_{1-6}\), retained the RPRL motif required for binding (Langelaan et al., 2009), although it is unclear if they maintain biological activity. Taken together, these findings may suggest that there is at least some common degradation pathway for [Pyr]apelin-13 in rats and humans \textit{in vivo}. Further studies are required to identify these proteases, as identification of more apelin degrading enzymes remains a crucial ongoing research area owing to the pivotal role of apelin in several physiological systems. Indeed, plasma kallikrein (KLKB1) was recently identified as another major enzyme that contributes to the rapid metabolism of apelin peptides – hydrolysing apelin-17 \textit{in vitro}
at its N-terminal to produce a C-terminal apelin-14 lacking the polar KFR head group (Fischer et al., 2019). However, the authors did not investigate whether or not the enzyme was able to hydrolyse [Pyr\(^1\)]apelin-13.

Inhibition of degradative enzymes is a well-established strategy to generate therapeutic agents. ACE2 is an essential member of the renin-angiotensin system, which converts angiotensin-II to angiotensin 1-7, with the latter mediating vasodilatation, anti-proliferation, anti-apoptosis and anti-fibrotic effects (Dai et al., 2015). In addition, ACE2 has been implicated in heart failure (Crackower et al., 2002; Yamamoto et al., 2006); diabetic nephropathy (Oudit et al., 2006; Tikellis et al., 2008); acute lung failure (Imai et al., 2005); lung injury induced by the lethal avian influenza A H5N1 virus (Zou et al., 2014); respiratory syncytial virus (Gu et al., 2016); and severe acute respiratory syndrome (SARS) (Imai et al., 2005). Recently, GlaxoSmithKline (GSK) developed a recombinant human ACE2, GSK2586881 for treatment of acute respiratory distress syndrome (ARDS) and showed that this molecule was well-tolerated in clinical trials (Khan et al., 2017). More recently, ACE2 was not only identified as the entry receptor for the causative virus of the current COVID19 pandemic (Hoffmann et al., 2020; Walls et al., 2020; Wan et al., 2020), but experimental studies in vascular and kidney organoid model suggested that recombinant human but not mouse soluble ACE2 was able to bind SARS-CoV-2 virus and prevent infection (Monteil et al., 2020). Corroborating on these, apelin signalling induces ACE2 expression in failing hearts (Sato et al., 2013), and protect against lung injury in experimental models of acute respiratory distress syndrome (Fan et al., 2015), possibly by inhibiting the NF-κB pathway and components of the inflammasome (Zhang et al., 2018b). Furthermore, apelin counteracts the effects of angiotensin-II signalling, which is negatively regulated by ACE2, suggesting that ACE2 and apelin could be a potentially novel therapeutic strategy for the treatment of lung injury related pathologies and heart failure.

The beneficial effects of apelin in heart failure are well characterised. Plasma apelin levels have been suggested to increase in early stages (Chen et al., 2003) of heart
failure but decreased in late stages of the disease (Chong et al., 2006; Földes et al., 2003; Francia et al., 2007). In support of this, heart failure therapies such as cardiac resynchronisation therapy used to treat refractory chronic heart failure were shown to increase plasma apelin, suggesting that increased apelin levels are associated with improved therapeutic benefit (Francia et al., 2007). Apelin administration increased stroke volume and contractility in failing hearts (Pitkin et al., 2010), thereby improving the performance of the failing heart. Similarly, neprilysin inhibitors have emerged as a pivotal therapeutic strategy for clinical management of heart failure due to the role of neprilysin in the degradation of vasoactive peptides including natriuretic peptides and bradykinin (Bavishi et al., 2015). Indeed, neprilysin inhibitors such as sacubitril are used for clinical management of heart failure (Velazquez et al., 2019). These data may therefore suggest that an additional benefit of neprilysin inhibitors in heart failure is to reduce apelin inactivation resulting in beneficial vasodilation, increased contractility and cardiac output.

Building on these findings, further studies could substitute the amino acids at the neprilysin cleavage sites in [Pyr₁]apelin-13 with unnatural amino acids to improve its resistance to degradation. Indeed, it was recently shown that infusion of neprilysin resistant apelin-17 in an established mice model of abdominal aortic aneurysm ameliorated the adverse aortic remodelling and aneurysm formation (Wang et al., 2019). Such a strategy was also demonstrated to significantly increase the resistance of [Pyr₁]apelin-13 and apelin-17 to ACE2 activity (Murza et al., 2014; Wang et al., 2016), suggesting that this could potentially be a mechanism to improve plasma stability of apelin-based therapeutics for clinical indications. We have recently published on another approach to stabilise apelin peptides in human blood using albumin domain (AlbudAb) antibody conjugated to an apelin analogue, MM202 and showed that this peptide was resistant to degradation yet retained biological activity at the human apelin receptor in vitro and in vivo (Read et al., 2019c). Therefore, these strategies could in the near future result in the development of the first apelin-based therapeutics for the treatment of human diseases.
In conclusion, apelin peptides have protective roles in cardiovascular diseases; however, any potential therapeutic use is impaired by the poor plasma stability of the peptide. In this study, a highly sensitive LC-MS/MS-based assay was developed for detection and quantification of [Pyr\textsuperscript{1}]apelin-13 in human plasma. For the first time in humans \textit{in vivo}, this study reports the identification of the most abundant metabolite of [Pyr\textsuperscript{1}]apelin-13, the ACE2 cleavage product, [Pyr\textsuperscript{1}]apelin-13\textsubscript{(1-12)} that we have previously demonstrated retains significant biological activity in addition to the putative neprilysin metabolites [Pyr\textsuperscript{1}]apelin-13\textsubscript{(4-13)} and [Pyr\textsuperscript{1}]apelin-13\textsubscript{(6-13)}. Combined inhibition of ACE2 and neprilysin may be a novel strategy to enhance endogenous apelin levels in conditions in which the peptide is downregulated, such as pulmonary arterial and essential hypertension. Additionally, these data will inform the design of more stable apelin peptides for therapeutic use in cardiorenal diseases.
Chapter 6  Pharmacology of Novel Apelin Analogues at the Apelin Receptor

6.1 Introduction

Apelin and its receptor are widely expressed in mammalian tissues including the heart, brain, lungs and kidney (Kleinz and Davenport, 2004, 2005; O’Carroll et al., 2013) and a number of physiological actions have been proposed for this peptide system (Read et al., 2019a; Yang et al., 2015). Importantly, cardiovascular and metabolic roles are the most characterised. In the cardiovascular system, apelin is a vasodilator (Japp et al., 2008; El Messari et al., 2004), strong positive inotrope (Berry et al., 2004; Chen et al., 2003; Maguire et al., 2009), and promotes angiogenesis. Apelin increases insulin sensitivity by promoting increased glucose uptake in skeletal muscle (Read et al., 2019a), and apelin knockout mice develop hyperinsulinemia and insulin resistance without any apparent changes in body weight (Dray et al., 2008; Yue et al., 2010). Additionally, in both standard and diet-induced obese mice, apelin decreased adiposity, serum insulin and triglycerides, without modulating food intake (Higuchi et al., 2007). These beneficial roles of apelin in regulating metabolism and energy expenditure have ignited interest in developing apelin analogues as a therapeutic strategy for improved management of metabolic diseases.

In order to further characterise the physiological effects of apelin in vivo, Galon-Tilleman et al. (Galon-Tilleman et al., 2017) generated a series of apelin-36 variants to understand the structure-activity relationship of the peptide and extend its plasma stability (Table 6.1). This series included a leucine-28 to alanine substitution (apelin-36-[L28A]) to test whether this site can tolerate further modifications, and subsequently a leucine-28 to cysteine substitution that allowed PEGylation of the resulting cysteine-28 (apelin-36-[L28C(30kDa-PEG)]), an established strategy to dramatically increase the plasma half-life of peptides by reducing proteolysis and renal excretion (Parrott
and DeSimone, 2012). They also tested a second site within the peptide, the C-terminal phenylalanine to alanine substitution (apelin-36-[F36A]) and its corresponding N-terminal PEGylated analogue, [40kDa-PEG]-apelin-36. Finally, they generated apelin-36-[A13 A28], which involves alanine substitutions at positions 13 and 28 (Galon-Tilleman et al., 2017). The authors reported that these peptides potently prevented diet-induced obesity by improving glucose tolerance, and decreasing blood glucose levels, cholesterol and LDL. However, surprisingly, they did not observe any effect on blood pressure in vivo, which would be expected for apelin receptor agonists (Galon-Tilleman et al., 2017). Apelin-36-[L28A] and apelin-36-[L28C(30kDa-PEG)] also showed impaired activation of the apelin receptor in a β-arrestin assay compared to apelin-36. The authors concluded that the metabolic action was independent of the apelin receptor and suggested that there could be another as yet unidentified receptor for apelin. However, the ability of these compounds to compete for binding of radiolabelled apelin was not determined.

Table 6.5. Sequences of apelin peptides showing amino acid residues affected by modifications in apelin-36.

<table>
<thead>
<tr>
<th>Peptides (Human)</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apelin-13</td>
<td>QRPRLSHKGPMVF (full agonist)</td>
</tr>
<tr>
<td>Apelin-36</td>
<td>LVQPRGSRNGPGWQGRRKFRQPRPRLSHKGPMVF (full agonist)</td>
</tr>
<tr>
<td>Apelin-36 (L28A)</td>
<td>LVQPRGSRNGPGWQGRRKFRQPRASHKGPMVF (100 fold less active at apelin receptor but full metabolic activity)</td>
</tr>
<tr>
<td>Apelin-36-[L28C(30kDa-PEG)]</td>
<td>LVQPRGSRNGPGWQGRRKFRQPRCys(30kDa-PEG)SHKGPMVF</td>
</tr>
</tbody>
</table>
Apelin-36 (F36A)  
LVQPRGSRNGPWPQGGRKFRQRPRLSHKGPMFA

Apelin-36 (A13 A28)  
LVQPRGSRNPGAWQGGRKFRQPRASHKGPMF

[40kDa-PEG]-Apelin-36  
(40kDa-PEG)LVQPRGSRNGPGWPQGGRKFRQRPRLSHKGPMF

The modifications are shown in bold. Comments refer to findings reported by Galon-Tilleman et al. (2017).

6.1.1 Aims and Hypotheses

These beneficial metabolic actions may represent a potential new target. Therefore, it is crucial to determine the mechanism of action and whether or not they are mediated via the apelin receptor. I hypothesised that these analogues were biased ligands of the apelin receptor. My aims were to:

1. Provide evidence for binding of these analogues to the apelin receptor in competition binding experiments in rat and the human heart.
2. Compare relative potencies of the analogues with apelin-36 in G protein-dependent (cAMP) and G protein independent (β-arrestin) in vitro assays.
3. Identify novel apelin receptor antagonists that could be used as tool compounds.
4. Investigate the activity of novel apelin analogues expected to have antagonistic properties.
6.2 Methods

6.2.1 Reagents and Chemical

[Apelin-36 and its analogues apelin-36-[F36A]], apelin-36-[L28A], apelin-36-[L28C(30kDa-PEG)], [40kDa-PEG]-apelin-36 and apelin-36-[A13 A28] were obtained from AstraZeneca (UK) Plc. The source of all other reagents as described in section 2.1.

6.2.2 Protein Extraction and Quantification

Samples of the human left ventricle were obtained from 5 dilated cardiomyopathy patients (4 males and 1 female, aged 38-55 years) undergoing transplantation. Rat hearts were obtained from adult male and female Sprague Dawley rats (n = 14; aged 7-10 weeks). Homogenates prepared as described in section 2.5.1 and resulting protein quantified by using Bio-Rad DC Protein Assay as described in section 2.5.2.

6.2.3 Saturation Binding Experiments

Saturation binding assays were performed using rat and human heart homogenate (n=3) by incubating with increasing concentrations of [125I]apelin-13 (2 nM-3.9 pM) as described in section 2.6.1. Binding in the presence of 2 µM [Pyr1]apelin-13 was used to define non-specific binding as this concentration will compete for >98% of total binding obtained with 2nM [125I]apelin-13.

6.2.4 Radioligand Competition Binding Assay

Competition binding experiments were performed using pooled human and rat heart homogenates (1.5 mg/ml) by incubating with [125I]apelin-13 (0.1 nM) in the presence of increasing concentrations of apelin-36, apelin-36-[L28A] or apelin-36-[L28C(30kDa-PEG)] (5x10^{-11}-10^{-4} M) as described in section 2.6.2. Binding in the presence of 2 µM [Pyr1]apelin-13 was considered non-specific.
6.2.5 Inhibition of cAMP Accumulation Assay

The cell-based cAMP assay was performed using Chinese Hamster Ovary cells (CHO-K1) cells artificially expressing the human apelin receptor, as described in section 2.7.1. Cells were stimulate with forskolin (15 µM) in presence of human [Pyr'1]apelin-13, human apelin-36, apelin-36-[F36A], apelin-36-[L28A], apelin-36-[L28C(30kDa-PEG)], apelin-36-[A13 A28] and [40kDa-PEG]-apelin-36 (1 pM-0.3 µM). Data were analysed in Graphpad Prism and values of potency, pD\textsubscript{2} (\text{-log}_{10} \text{EC}_{50}, where \text{EC}_{50} is the concentration producing half-maximal response) determined.

6.2.6 β-Arrestin Recruitment Assay

β-arrestin recruitment assays were performed using CHO-K1 cells artificially expressing the human apelin receptor. Cells were incubated with increasing concentrations of human [Pyr'1]apelin-13, human apelin-36, apelin-36-[F36A], apelin-36-[L28A], apelin-36-[L28C(30kDa-PEG)], apelin-36-[A13 A28] and [40kDa-PEG]-apelin-36 as described in section 2.7.2. Data were analysed in GraphPad Prism 6, and values of pD\textsubscript{2} and maximum response (E\textsubscript{Max}) were calculated for each compound.

6.2.7 Statistical Analysis

Data were expressed as mean±SEM. Experiments were performed in triplicates or duplicates, and n-values were described as the number of independent experiments. Potencies of analogues in cell-based assays were compared to native apelin-36 using a one-way ANOVA with Dunnett's posthoc test in GraphPad Prism 6. A P value < 0.05 was considered statistically significant. Binding affinities in both species were compared using Student's t-test in GraphPad Prism as described above. Bias calculations were performed as previously described using the operational model for bias (van der Westhuizen et al., 2014), to obtain values for the relative effectiveness of the apelin-36 synthetic analogues compared to apelin-36 within each cell-based assay. Bias factors were calculated to compare the relative activities of the analogues.
between the different pathways – β-arrestin (G protein independent) and cAMP (G protein-dependent).
6.3 Results

6.3.1 Characterisation of [Pyr¹]Apelin-13 Binding in Human and Rat Heart

In human and rat heart, [Pyr¹]apelin-13 bound with a comparable single Hill slope close to 1 (human 0.95±0.11, rat 0.95±0.03), and subnanomolar affinities (human \( K_D \), 0.08±0.01 nM; rat \( K_D \), 0.28±0.02 nM) for the apelin receptor. The apelin receptor density in both species was also similar (human, 13.82±1.79 fmol/mg; rat, 83.4±1.85 fmol/mg) (Fig.1).
Figure 6.1. Characterisation of binding of $[^{125}\text{I}]$apelin-13 to rat and human cardiac apelin receptors. $[^{125}\text{I}]$-apelin-13 saturation binding in human (A) and rat (B) homogenates (data are mean±SEM). NSB is nonspecific binding defined in the presence of 2 µM [Pyr']apelin-13.
6.3.2 Apelin-36, Apelin-36-[L28A] and Apelin-36-[L28C(30kDa-PEG)] Compete for Binding to the Apelin Receptor in Rat and Human Heart

In rat hearts apelin-36 (pKᵢ, 10.23±0.12) and apelin-36-[L28A] (pKᵢ 9.51±0.18) bound the receptor with subnanomolar affinities, and apelin-36-[L28C(30kDa-PEG)] with low nanomolar affinities (pKᵢ, 8.06±0.37) (Fig. 6.2A,C,E). Similarly, apelin-36 bound the human apelin receptor with subnanomolar affinity (pKᵢ: apelin-36 10.28±0.09) but its analogues apelin-36-[L28A] and apelin-36-[L28C(30kDa-PEG)] competed for apelin receptor binding in human heart with low nanomolar affinities (apelin-36-[L28A], 8.52±0.05; apelin-36-[L28C(30kDa-PEG)], 8.00±0.05), (Fig. 6.2B,D,F). Whereas the PEGylated apelin-36 affinity was not different for human and rat receptors, apelin-36-[L28A] had a significant 10-fold higher affinity for the rat receptor (p<0.05).
Figure 6.2. Relative affinity of apelin-36 analogues in rat and the human heart. Apelin-36 and its analogues, apelin-36-[L28A], apelin-36-[L28C(30kDa-PEG)] bind to the apelin receptor in rat and the human heart. Apelin-36 (A) Apelin-36-[L28A] (C), apelin-36-[L28C(30kDa-PEG)] (E) binding in rat heart homogenates. Apelin-36 (B) Apelin-36-[L28A] (D) and apelin-36-[L28C(30kDa-PEG)] (F) binding in human heart homogenates. Data are mean±SEM; $pK_i = -\log_{10} EC_{50}$ of equilibrium dissociation constant determined in a competition binding experiment ($K_i$).
6.3.3 The Activity of Apelin-36 Analogues in the cAMP Assay

In the cAMP assay, the rank order of potencies was apelin-36 > apelin-36-[L28A] ≥ apelin-36-[L28C(30kDa-PEG)] (pD$_2$: 9.04±0.45 > 7.88±0.24 ≥ 7.02±0.09, respectively). Both analogues were less potent than apelin-36 but this was significant only for apelin-36-[L28C(30kDa-PEG)] (p<0.001), (Fig. 6.3, Table 6.2). [40kDa-PEG]-apelin-36 had similar potency to the endogenous apelin-36 in the cAMP assay (pD$_2$ 8.69±0.22) (Fig. 6.3C; Table 6.2). Similarly, pD$_2$ values for apelin-36-[F36A] and apelin-36-[A13 A28] were comparable to apelin-36 in this assay (pD$_2$ 8.90±0.21 and 8.12±0.34, respectively).

Figure 6.3. Relative potency of apelin-36 analogues in cell-based assays. The apelin isoforms, [Pyr$^1$]apelin-13 and apelin-36 were tested in cell-based β-arrestin and cAMP assays. β-arrestin data are shown in A,C; cAMP data are shown in B,C. Data are mean±SEM of 3-4 independent experiments. pD$_2$ (negative log$_{10}$ of the concentration producing 50% of maximum response) from each peptide was compared to apelin-36 using one-way ANOVA. P < 0.05 was considered statistically significant.
Table 6.2. Summary of the relative potency of apelin-36 analogues.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>β-Arrestin Assay</th>
<th>cAMP Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC$_{50}$ (nM)</td>
<td>pD$_2$±SEM</td>
</tr>
<tr>
<td>[Pyr$^1$]apelin-13</td>
<td>2.75</td>
<td>8.56±0.17</td>
</tr>
<tr>
<td>Apelin-36</td>
<td>0.68</td>
<td>9.17±0.34</td>
</tr>
<tr>
<td>Apelin-36-[L28A]</td>
<td>37.2</td>
<td>7.43±0.07$^{***}$</td>
</tr>
<tr>
<td>Apelin-36-[L28C(30kDa-PEG)]</td>
<td>1900</td>
<td>6.05±0.06$^{***}$</td>
</tr>
<tr>
<td>Apelin-36-[F36A]</td>
<td>8.18</td>
<td>8.09±0.25$^{	ext{**}}$</td>
</tr>
<tr>
<td>Apelin-36-[A13 A28]</td>
<td>150</td>
<td>6.82±0.14$^{***}$</td>
</tr>
<tr>
<td>[40kDa-PEG]-Apelin-36</td>
<td>1.95</td>
<td>8.71±0.15</td>
</tr>
</tbody>
</table>

Data are mean±SEM n = 3-4 independent experiments. pD$_2$ (negative log$_{10}$ of the concentration producing 50% of maximum response). $^{	ext{*}}$Significantly different from Apelin-36 compared using ANOVA with Dunnett’s posthoc test and significance set at $p < 0.05$ (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

### 6.3.4 The activity of the apelin-36 analogues in β-arrestin recruitment assays

In the β-arrestin assays, the lower potency obtained with apelin-36-[L28A] (pD$_2$ 7.43±0.07) and apelin-36-[L28C(30kDa-PEG)] (pD$_2$ 6.05±0.06) compared to apelin-36 (pD$_2$ 9.17±0.34) was more apparent than in the cAMP assay, with both analogues being significantly less potent than apelin-36 (p<0.01; Fig. 6.3B, Table 6.2). Similarly,
when compared to apelin-36 (pD₂ 9.17±0.34) responses to apelin-36-[F36A] (pD₂ 8.09±0.25, p<0.01) and apelin-36-[A13 A28] (pD₂ 6.82±0.14, p<0.0001) were significantly decreased (approximately 12- and 221-fold respectively) in this assay (Fig 6.3D, Table 6.2). [40kDa-PEG]-apelin-36 had similar potency to the endogenous apelin-36 in the β-arrestin assay (pD₂; 8.71±0.15 vs 9.17±0.34) (Fig 6.3D, Table 6.2).

6.3.5 The Apelin-36 Analogues Show Biased Signalling at the Apelin Receptor

Apelin-36 and [40kDa-PEG]-apelin-36 each exhibited similar potency in the β-arrestin compared to cAMP assays although the PEGylated apelin-36 demonstrated a small drop in potency compared to the native peptide (Table 6.2). However, apelin-36[L28A], apelin-36-[L28C(30kDa-PEG)], apelin-36[F13A] and apelin-36-[A13 A28] were approximately 3-, 10-, 12 and 20-fold more potent in the cAMP assay compared to the β-arrestin assay respectively (Fig. 6.3A,B; Table 6.2). The degree of functional selectivity (bias) of these apelin-36 analogues for the two pathways, G protein-dependent (cAMP assay) and G protein-independent (β-arrestin assay), was calculated as previously described (van der Westhuizen et al., 2014), using apelin-36 as the reference ligand for this analysis. The relative effectiveness (RE), transduction ratio (logR) and bias factors are shown in Tables 6.3 and 6.4 with apelin-36[L28C-(30kDa-PEG)] showing the greatest degree of preference for the G protein pathway. These data are confirmed in the bias plot (Figure 6.4) (Kenakin et al., 2012) that further indicates that these analogues are G protein biased at the level of the receptor in a system-independent manner.
Table 6.3. Values of $\Delta \log R$ and relative effectiveness (RE) for apelin-36 analogues compared to apelin-36 in cAMP and $\beta$-arrestin assays.

<table>
<thead>
<tr>
<th>Agonists</th>
<th>cAMP Assay</th>
<th></th>
<th>$\beta$-Arrestin Assay</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta \log R$</td>
<td>RE</td>
<td>$\Delta \log R$</td>
<td>RE</td>
</tr>
<tr>
<td>[Pyr$^1$]apelin-13</td>
<td>0.10±0.35</td>
<td>1.26</td>
<td>-0.82±0.13</td>
<td>0.15</td>
</tr>
<tr>
<td>Apelin-36</td>
<td>0.00±0.43</td>
<td>1.00</td>
<td>0.00±0.28</td>
<td>1.00</td>
</tr>
<tr>
<td>Apelin-36-[L28A]</td>
<td>-1.06±0.35</td>
<td>0.09</td>
<td>-2.18±0.17</td>
<td>0.01</td>
</tr>
<tr>
<td>Apelin-36-[L28C-30kDa-PEG]</td>
<td>-1.95±0.31</td>
<td>0.01</td>
<td>-3.71±0.12</td>
<td>0.0002</td>
</tr>
<tr>
<td>Apelin-36-[F36A]</td>
<td>0.25±0.33</td>
<td>1.78</td>
<td>-1.19±0.23</td>
<td>0.06</td>
</tr>
<tr>
<td>Apelin-36-[A13 A28]</td>
<td>-1.06±0.27</td>
<td>0.09</td>
<td>-2.28±0.15</td>
<td>0.01</td>
</tr>
<tr>
<td>[40kDa-PEG]-Apelin-36</td>
<td>0.08±0.40</td>
<td>1.20</td>
<td>-0.59±0.16</td>
<td>0.26</td>
</tr>
</tbody>
</table>

$\Delta \log R$ is the $\log_{10}(\tau/K_A)$ where $\tau$ is a measure of agonist efficacy, and $K_A$ is a measure of functional affinity (van der Westhuizen et al., 2014); for each assay $n=3-4$ independent experiments.
Table 6.4. $\Delta\Delta\log R$ and bias factor for apelin-36 analogues compared to apelin-36 in cAMP and $\beta$-arrestin assays.

<table>
<thead>
<tr>
<th>Agonists</th>
<th>cAMP vs $\beta$-arrestin Assay</th>
<th>$\Delta\Delta\log R \pm $SEM</th>
<th>Bias factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Pyr$^1$]apelin-13</td>
<td></td>
<td>0.92$\pm$0.37</td>
<td>8</td>
</tr>
<tr>
<td>Apelin-36</td>
<td></td>
<td>0.00$\pm$0.51</td>
<td>1</td>
</tr>
<tr>
<td>Apelin-36-[L28A]</td>
<td></td>
<td>1.12$\pm$0.39</td>
<td>13</td>
</tr>
<tr>
<td>Apelin-36-[L28C-30kDa-PEG]</td>
<td></td>
<td>1.76$\pm$0.33**</td>
<td>58</td>
</tr>
<tr>
<td>Apelin-36-[F36A]</td>
<td></td>
<td>1.44$\pm$0.40*</td>
<td>28</td>
</tr>
<tr>
<td>Apelin-36-[A13 A28]</td>
<td></td>
<td>1.23$\pm$0.31*</td>
<td>17</td>
</tr>
<tr>
<td>[40kDa-PEG]-Apelin-36</td>
<td></td>
<td>0.67$\pm$0.43</td>
<td>5</td>
</tr>
</tbody>
</table>

$\Delta\Delta\log R$ is the difference between $\Delta\log R$ values in both pathways (cAMP or G protein and $\beta$-arrestin pathways). *Significantly different from Apelin-36 compared using Student’s t-test with significance set at $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$).
Figure 6.4. Bias plot for apelin-36 and analogues in cAMP and β-arrestin assays. Curves show the corresponding responses in each assay to equivalent concentrations of apelin-36 and analogues in CHO-K1 cells expressing the apelin receptor. Deviation in the shape of the curves indicates ligand bias at the receptor level. Responses in the cAMP assay were expressed as % inhibition of forskolin response; in the β-arrestin assay as % of the maximal response to [Pyr¹]apelin-13.
6.4 Discussion

This study report on the pharmacodynamic characteristics of apelin-36 analogues that were designed to have more extended plasma stability, some of which were proposed to exert apelin receptor-independent effects (Galon-Tilleman et al., 2017). I have now demonstrated that apelin-36-[L28A] and apelin-36-[L28C(30kDa-PEG)] do bind to the apelin receptor in human and rat heart where they competed for binding with [125I]apelin-13 with nanomolar affinities. These data, therefore, imply that the reported beneficial metabolic mechanism of action for these analogues is likely through the apelin receptor. Compared with the sub-nanomolar affinity of apelin-36 in the heart from both species, the apelin-36 L28A mutation resulted in an order of magnitude reduction in affinity. This was further reduced in the PEGylated analogue and may be explained by the general steric hindrance in the bulky PEGylated form. Mutations at the L5A, position in apelin-13 (corresponding to L28A in apelin-36) had a modest effect on apelin receptor binding and signalling in cultured cells stably expressing the receptor (Fan et al., 2003; Medhurst et al., 2003). Our data for the apelin-36 analogues in experiments using native rat and human receptor confirm that the mutation at this position in the longer apelin isoform does not adversely affect binding affinity for the apelin receptor.

In cell-based assays, I confirmed the decreased β-arrestin activation initially reported by Galon-Tilleman et al. (Galon-Tilleman et al., 2017), who found that apelin-36-[L28A] and apelin-36-[L28C(30kDa-PEG)] were 100 and 10,000-fold respectively less potent compared to the endogenous apelin-36, although in this study the reduction in potency of apelin-36-[L28C(30kDa-PEG)] was only 1400-fold. I have now determined the potency of these analogues and found them to be less effective than apelin-36 in both the G protein-dependent cAMP accumulation and β-arrestin assays. However, this potency reduction was more apparent in the β-arrestin assay indicating a degree of G protein bias for these analogues compared to apelin-36. Further analysis confirmed both were G protein, biased agonists, with bias factors of 13 and 58, respectively.
However, it is also possible that apelin-36-[L28C(30kDa-PEG)] may be a partial agonist at the apelin receptor, and not necessarily bias considering that it did not produce a maximum response in the β-arrestin assay.

In addition, alanine substitutions of proline and leucine at positions 13 and 28, apelin-36-[A13 A28], resulted in an approximately 10-fold decrease in potency in the β-arrestin assay compared to cAMP assay. The bias factor for this peptide was 17, suggesting that alanine substitution at these positions promote G protein signalling over β-arrestin recruitment. Hence, these findings are consistent with enhanced functional selectivity (bias) towards G protein-dependent signalling by these apelin-36 analogues. We have previously reported that the apelin receptor is tractable to development of biased agonists and identified a biased apelin peptide, MM07, generated by N-terminal cyclisation with flanking cysteine residues (Brame et al., 2015; Yang et al., 2019). Recently, we reported on the identification of the first small molecule biased apelin receptor agonist, CMF-019 (Read et al., 2016).

Molecular dynamic simulation and site-directed mutagenesis showed that upon ligand binding to the apelin receptor, the C-terminal phenylalanine residue of apelin peptides is embedded by Trp\textsuperscript{152} in the transmembrane domain IV and by Trp\textsuperscript{259} and Phe\textsuperscript{255} in transmembrane domain VI of the apelin receptor. Deletion of this amino acid abrogated β-arrestin-mediated apelin receptor internalisation but did not affect cAMP inhibition (Iturrioz et al., 2010). Genetic inactivation of β-arrestin recruitment and signalling at the apelin receptor inhibited ligand-induced receptor internalisation and protected mice from diet-induced obesity by decreasing body weight, adiposity and improving glucose tolerance and insulin sensitivity without affecting food intake or cardiovascular function (Li et al., 2020a). Furthermore, we and others have also shown that deletion of the phenylalanine residue does not affect binding and that the resulting peptide is still functional at the receptor (Wang et al., 2016; Yang et al., 2017c). Consistent with this, alanine substitution of the apelin-36 C-terminal phenylalanine, apelin-36[F13A], biased responses towards G protein-dependent signalling with a bias
factor of 28. Taken together, the beneficial metabolic actions of apelin receptors may be mediated by G protein-dependent but β-arrestin-independent signalling.

Apelin receptor-mediated metabolic effects have been well characterised. For example, apelin inhibited isoproterenol-induced free fatty acid release in isolated adipocytes (Yue et al., 2011). The apelin effect was attenuated by inhibition of Gαq with Gp2A or Gαi with pertussis toxin or AMP-activated protein kinase (AMPK) by either compound C or dorsomorphin (Yue et al., 2010, 2011). Apelin also stimulates glucose transport in skeletal muscle, and adipose tissue via the activation of AMPK and eNOS demonstrated using mice expressing a muscle-specific dominant-negative AMPK mutation (Attane et al., 2011; Dray et al., 2008, 2013; Yue et al., 2010). Recently, in a diet-induced obesity mouse model, it was shown that acute administration of apelin-13 and analogues with enhanced in vitro plasma stability resulted in improved glucose homeostasis and insulin secretion (O'Harte et al., 2017, 2018). Similarly, in the same model, chronic administration of [Pyr1]apelin-13 systemically over 28 days, exhibited anti-diabetic properties by directly targeting lipid metabolism, thus reducing hepatic steatosis (Bertrand et al., 2018). This was likely a result of improved insulin sensitivity and glucose uptake by the muscle rather than a direct effect on hepatocytes.

There is still an unmet need for the development of therapeutics that could improve the management, and potentially treat type 2 diabetes mellitus and its associated metabolic syndromes. This is particularly important since over 415 million people globally suffer from type 2 diabetes mellitus, and the figure is expected to rise to 642 million by 2040 (Zimmet et al., 2016). The apelin receptor signalling pathway has been shown to modulate several physiological and pathological states. It has acute beneficial effects in cardiovascular and metabolic diseases including myocardial infarction (Berry et al., 2004; Japp and Newby, 2008; Simpkin et al., 2007), hypertension (Brame et al., 2015; Przewlocka-Kosmala et al., 2011; Yang et al., 2017b), obesity and type 2 diabetes (Bertrand et al., 2015; Castan-Laurell et al., 2011). This suggests that apelin-based therapeutics may present a novel mechanism for
clinical management or treatment of the metabolic syndrome. However, like most hormonal peptides their activity is often short-lived owing to rapid degradation by endopeptidases. This has been the impetus to develop apelin peptides with improved pharmacokinetic properties. This study, therefore, reinforces this by demonstrating that the beneficial effects of these analogues in diet-induced obesity mice model were mediated via the apelin receptor, making it a novel therapeutic target in diabetes.

**6.4.1 Conclusion**

Although initially reported to mediate their effects in an apelin receptor-independent manner, this study have now shown that apelin-36-[L28A] and apelin-36-[L28C(30kDa-PEG)] bind to the apelin receptor with nanomolar affinities. The data presented in this study provide evidence that these peptides are G protein biased apelin receptor agonists, and this pharmacological profile is consistent with their reported beneficial *in vivo* metabolic actions via Gαi and Gαq signalling pathways (Fig. 6.5). However, a potential partial agonism of the analogues at the apelin receptor can not be excluded.
Figure 6.5. Proposed signalling cascade activated by apelin-36-[L28A] and apelin-36-[L28C(30kDa-PEG)] upon binding to the apelin receptor. On the left, apelin-36 recruits Gαi and Gαq signalling following receptor binding resulting in the activation of AMP-dependent kinase. However, it will also activate β- arrestin recruitment, which will result in receptor desensitisation and degradation or recycling back to the cell surface. On the other hand, apelin-36-[L28A] and apelin-36-[L28C(30kDa-PEG)] on the right recruits Gαi and Gαq signalling resulting in the activation of AMP-dependent kinase group of enzymes which mediates the downstream beneficial metabolic effects. There are reduced receptor internalisation and desensitisation, which is likely to prolong the agonist activity of the two peptides compared to apelin-36.
Chapter 7  General Conclusions

7.1  Apelin, ELA and Apelin Receptor in the Kidney

Apelin was considered to be the only ligand of the apelin receptor until the discovery of ELA in 2013. In zebrafish, ELA was not only identified as the earliest endogenous ligand of the apelin receptor but also crucial in cardiovascular development. However, recent studies in mice demonstrated that apelin, not ELA, was the first expressed endogenous ligand of the apelin receptor in mammals (Freyer et al., 2017), although there were phenotypic differences between apelin and apelin receptor mutants that might be explained by the presence of the second ligand, ELA. In adult rodents, ELA expression was proposed to be restricted to the kidney and was shown to promote diuresis (Deng et al., 2015). However, ELA also produced significant cardiovascular effects similar to apelin (Yang et al., 2017b), suggesting a distribution of this peptide beyond the kidney. Therefore, the first aim of this thesis was to determine the expression of ELA relative to apelin and apelin receptor in mouse and human tissues. The precise cellular distribution of ELA, apelin and apelin receptor in the human kidney was also explored. Genetic deletion of specific genes is a widely used and effective method to decipher physiological functions. Thus, the interaction of ELA and apelin, and their common apelin receptor was further explored by investigating changes, if any, in apelin and apelin receptor levels in mice deficient for ELA.

7.1.1 Expression Profile of ELA Relative to Apelin and Apelin Receptor

This study demonstrated that ELA was expressed at the mRNA level in several tissues, including the heart, kidney, brain, and lungs in both mouse and humans. This finding significantly expands on the previous study where ELA mRNA was found in the non-cardiomyocyte component of the heart (Perjés et al., 2016). ELA and apelin mRNA were not present in the spleen and liver despite the presence of the apelin receptor mRNA, indicating a potential endocrine role of apelin and ELA in these
tissues. Consistent with the initial reports that in rodents ELA was exclusively expressed in the kidney (Deng et al., 2015), in this study mouse kidney had the highest level of ELA, which was over 85-fold higher than apelin. However, this was not replicated in the human kidney, where although present ELA levels were not significantly different from apelin or apelin receptor, thereby suggesting a marked species difference. This may support the hypothesis that in rodents, ELA was predominantly synthesised in the kidney and secreted into circulation to act on distant tissues whereas, in humans, ELA was locally produced, suggesting that the endothelium was possibly the main source of circulating ELA in humans (Yang et al., 2017b). Therefore, these results may have implications for the relevance of rodent models for assessment of the physiological role of the apelin system in the human kidney or to test the therapeutic potential of apelin compounds.

The cellular distribution of apelin, ELA, and apelin receptor in the human kidney, especially at the protein level, is unknown. This study demonstrated that both ligands and their receptor were expressed from the glomerulus to the collecting duct (Fig. 7.1), consistent with a potentially important role of the apelin system in the regulation of renal physiology. The localisation of ELA in the apical membrane (lumen) of the renal nephron suggested that it was likely to be secreted in urine. Indeed, a recent study confirmed this by measuring high levels of ELA in urine in humans (Zhou et al., 2019). Although the role of ELA in urine is unknown, this may support a potential paracrine role in the activation of apelin receptors in distant segments of the renal nephron. On the other hand, apelin was localised to the basolateral membrane only, and this may explain why it has never been detected in the urine. Regardless, both apelin and ELA were present in renal epithelial cells suggesting that in addition to the endothelium previously shown to produce these peptides (Yang et al., 2017b), they can also be made by epithelial cells in the kidney.
This study, for the first time, demonstrated that ELA mRNA was widely expressed beyond the kidney alone. A potential species-difference was observed in the expression of ELA mRNA in human and mouse kidney. Surprisingly, although the apelin receptor was present in the spleen and liver, the ligands were absent in these tissues. At the protein level, this study showed that ELA, apelin and apelin receptor were localised from the glomerulus to the collecting duct in the renal nephron. Notably, the apelin receptor was expressed in the juxtaglomerular apparatus, suggesting that it could respond to circulating ELA and apelin, and potentially regulate systemic blood pressure. Further studies, including a micropuncture experiment on isolated nephron or nephron segments, are needed to further determine the functions of the apelin system in the kidney. Additionally, although expression of ELA, apelin and apelin

Figure 7.1. Renal nephron showing localisation of the apelinergic system. LoH, the loop of Henle; proximal straight tubule is part of the proximal convoluted tubule; (image generated from smart_servier medical art).
receptor was observed in the other tissues including brain and lungs, it remains to be determined which cell type they are expressed in, especially in the brain that comprises anatomically diverse regions.

### 7.1.2 Clinical Relevance of Apelin Pathway in Kidney Disease

I next, addressed the clinical relevance of the apelin system in kidney disease. In a mouse model of chronic kidney disease, both apelin and ELA mRNA were significantly decreased, but the receptor was unaffected. Consistent with this observation, both ELA and apelin has previously been shown to prevent kidney injury and fibrosis, critical hallmarks of chronic kidney disease (Chen et al., 2015a, 2017; Huang et al., 2018). Therefore, exogenous ELA or apelin peptides may replace the downregulated peptide and provide clinical benefit in patients with chronic kidney disease.

Evaluation of circulating ELA and apelin levels in CKD patients revealed that plasma levels of these peptides were increased compared to control. Interestingly, the circulating levels of both apelin and ELA were negatively correlated with eGFR, a surrogate measure of renal function, indicating that levels of these peptides increased as the disease worsened possibly as a compensatory mechanism to restore renal function. In support, levels of apelin and ELA in these patients were positively associated with plasma levels of endothelin-1 (ET-1), big ET-1, big ET-1: ET-1 ratio and asymmetric dimethylarginine, a nitric oxide synthase inhibitor (Chapman et al., 2020). Moreover, a recent study demonstrated that the serum levels of apelin were significantly increased in haemodialysis and patients with stage 3-5 CKD compared to healthy controls (Trojanowicz et al., 2020). They further showed that leucocytes isolated from the blood of haemodialysis and CKD3-5 patients but not controls had upregulated mRNA expression of AT$_1$R and AT$_2$R, which correlated with decreased ACE2 mRNA levels. Interestingly, treatment of patient-derived monocytes with apelin downregulated the expression of inflammatory markers including interleukin-6 and tumour necrosis factor-α (TNFα), as well as the angiotensin receptors, AT$_1$R and AT$_2$R, but upregulated ACE2 levels without affecting ACE expression (Trojanowicz et
Taken together, these studies support a renoprotective role for the apelin system in kidney disease.

Furthermore, a recent observational study of ELA in a small population (n=20) with diabetes nephropathy reported decreased circulating levels of the peptide with disease and that ELA levels were positively associated with eGFR (Zhang et al., 2018a), a measure of kidney function. Apelin levels were also previously suggested to decrease in chronic kidney disease patients on dialysis (Matyszko et al., 2006). The discrepancy between these previous studies and the data presented here and by Chapman et al., (2020) may be due to: advanced disease pathology in the two studies compared to this study, and, that the underlying aetiology of the chronic kidney diseases in patients studied here were diverse (see Chapman et al., 2020). In support of the first point, it was shown that in the initial stages of heart failure, apelin levels are elevated in an attempt to minimise cardiac damage and protect the heart but massively decreased in more advanced disease (Japp and Newby, 2008). Therefore, this study demonstrated that targeting the apelin pathway might be a potentially novel mechanism to treat renal diseases such as CKD.

7.1.3 Effect of ELA Deletion on Apelin, Apelin Receptor and the RAS System

Having established that ELA expression was not limited to the kidney, described the cellular distribution of the apelin system in human kidney and its clinical potential in CKD, I sought evidence for an interaction between ELA and apelin or apelin receptor using Apela null mice. In this study, a potentially tissue-specific effect of ELA on apelin and apelin receptor was observed. In the brain, Apela deletion did not significantly alter the levels of Aplnr or Apln, but there was a trend for decreased Apln. Also, loss of Apela in the heart appeared to upregulate Apln and Aplnr expression, but this was not statistically significant, whilst in the kidney where Apela was the most abundant of the three genes, there was a statistically significant downregulation of Apln but not the Aplnr expression following Apela deletion. In support, a tissue-specific compensatory
effect of apelin in the absence of ELA is plausible in the heart and kidney. Both apelin and ELA increased cardiac contractility, stroke volume and cardiac output ex vivo and in anaesthetized animals, suggesting that at least in this organ similar downstream signalling pathway was activated by both ligands (Murza et al., 2016; Read et al., 2019a).

Moreover, at the kidney level, both apelin and ELA promoted diuresis when infused into rodents (Deng et al., 2015; Hus-Citharel et al., 2008, 2014; Murza et al., 2016; Schreiber et al., 2016). The mechanism of apelin-induced diuresis involved inhibition vasopressin-mediated aquaporin-2 translocation to the apical membrane (Hus-Citharel et al., 2014), but that of ELA remains to be explored. However, apelin was not only unable to rescue the phenotype of Apela knockout mouse placenta, but treatment of apelin receptor-expressing primary allantois cultures with apelin or ELA resulted in opposing effects on the expression of the tip cell gene, esm1 and hypoxia response genes (Ho et al., 2017). This, therefore, suggests that apelin and ELA can also activate distinct signalling pathways downstream the apelin receptor in cell type or tissue-specific manner.

Next, I investigated the effect of ELA knockout on components of the RAS, since the apelin receptor signalling pathway negatively modulates RAS. One area where this has been well described is failing hearts where apelin and ELA signalling negatively regulate RAS to provide cardioprotection (Iwanaga et al., 2006; Sato et al., 2013, 2017; Siddiquee et al., 2013). One of the ways ELA signalling protects the heart from damage involves direct inhibition of ACE transcription factor, FOXM1, resulting in the downregulation of ACE expression (Sato et al., 2017). However, an association between ELA signalling and ACE or ACE2 in the kidney has not been previously described. This study found no difference in ACE/ACE2 ratio in cardiac tissues. However, there was a significantly increased ACE/ACE2 ratio in the kidney of ELA deficient mice, suggesting that ELA may also regulate ACE levels in physiological conditions.
ACE/ACE2 ratio is often used as a surrogate measure of the RAS activation and increased circulating angiotensin II, which promotes kidney injury/damage, vasoconstriction, inflammation, and fibrosis (Bernardi et al., 2012; Koka et al., 2008; Wang et al., 2011; Yang et al., 2017a). Therefore, one of the roles of ELA in the kidney may be to suppress activation of intra-renal ACE expression, thereby protecting the kidney from injury and disease. Consistent with this hypothesis, a recent study suggested that ELA inhibited intra-renal RAS activation induced by high salt loading in Dahl-salt sensitive rats via decreased expression of renin, angiotensinogen, angiotensin receptor types 1 and 2 (AT₁R and AT₂R). Interestingly, the levels of ACE2 were upregulated by ELA administration, and this was accompanied by attenuated hypertension, fibrosis, kidney injury, and inflammation (Xu et al., 2020).

Taken together, this part of my study suggested that genetic loss of ELA might have a tissue-specific effect on the expression of apelin and apelin receptor at least in mice. Additionally, ELA deletion affected key components of the RAS pathway. Hence, ELA may protect the kidney from injury and damage by regulating levels of renal ACE and ACE2, thereby contributing to the homeostatic control of the detrimental angiotensin II and the beneficial angiotensin 1-7 signalling.

### 7.2 Processing of ELA and Apelin in Humans

Following the discovery of ELA, two other isoforms, ELA-21 and ELA-11 were predicted to form because of cleavage by furin. However, this has not been shown experimentally elsewhere. Also, previous metabolism studies of apelin were performed either *in vitro* or *in vivo* in animal models (Mesmin et al., 2010; Onorato et al., 2019), but no studies have previously investigated the metabolism of apelin peptides *in vivo* in humans and identify the metabolites generated. Hence, the second aim of this thesis was to seek experimental evidence for the production of ELA-21 and ELA-11 from mature ELA-32 peptide *in vitro* in human plasma and kidney
homogenates and identify other cleavage sites and shorter isoforms of the peptide. The most abundant endogenous ELA peptides generated in human tissues was also sought. Understanding this is necessary for the development of protease-resistant analogues of ELA as tool compounds for experimental medicine studies and potentially treatment of cardiorenal diseases. Additionally, the metabolism of \([\text{Pyr}^1]\text{apelin-13 in vivo}\) in humans was investigated and its metabolites identified.

### 7.2.1 Metabolism of Mature ELA-32 and Identification of Endogenous ELA Isoform

Here, I have demonstrated that ELA-32, although longer and should potentially have more proteolytic cleavage sites, had a longer plasma half-life (>40 mins) when compared to \([\text{Pyr}^1]\text{apelin-13}, \text{whose half-life was <5 mins}\) (Mesmin et al., 2010; Murza et al., 2016). The significant difference in plasma stability may provide a clue to why two ligands activate the same receptor and suggest that perhaps, under physiological conditions, the more stable ELA may take over the roles of degraded apelin. In support, circulating ELA levels were not only higher than that of apelin (Yang et al., 2017b), but there was also a strong positive correlation between circulating apelin and ELA levels in humans, especially in a disease state such as CKD. Additionally, the proteolytic cleavage of ELA-32 in human plasma produced over ten other isoforms, including ELA-11, ELA-16, ELA-18, ELA-19, ELA-20 and ELA-22, but ELA-21 was not detected (Fig. 7.2). Both ELA-16 (has a disulphide bridge) and ELA-14 had been suggested to retain similar potency and affinity for the apelin receptor as the longer isoform, ELA-32. Notably, ELA-14 decreased mean arterial pressure and increased cardiac contractility and cardiac output both \textit{in vivo} and in isolated hearts (Murza et al., 2016). Hence, the degradation of ELA-32 in human plasma to these isoforms may preserve biological function.
Figure 7.2. Proposed degradation profile of ELA-32 in human plasma. The degradation study suggested that the longer isoforms were sequentially cleaved to generate shorter isoforms during the 240 mins incubation. ELA-21 was not detected in either human plasma or kidney, but ELA-22 and ELA-20 were detected. Red | indicated cleavage point; amino acid residues critical for receptor binding are shown in magenta.

To date, the enzyme(s) involved in the degradation or cleavage of ELA into shorter isoforms have not been identified. Although ELA-32 contains potential furin cleavage sites, the exact furin responsible for its metabolism is unknown. In the case of apelin, the furin, PCSK3 was shown to convert apelin-55 to apelin-13 (Shin et al., 2019). In this study, two peptides potentially generated by ACE2 activity were identified. These include ELA-16/17 and ELA-19/17, both of which were produced after initial proteolytic removal of the C-terminal Pro$^{32}$. However, further studies are required to demonstrate that ACE2 can metabolise ELA into these fragments.
ELA-11 peptide was identified in zebrafish embryos overexpressing ELA mRNA by mass spectrometry (Pauli et al., 2014), following the initial discovery of ELA. However, the endogenous levels of the longer isoforms have not been previously reported. To detect and quantify endogenous ELA peptides, I have developed an extraction method amenable to mass spectrometry. Using this method, endogenous ELA-11 was detected in human tissues on a highly sensitive mass spectrometer, but the longer isoforms were undetectable. A previous study did not only indicate that ELA-32 was cleaved to ELA-11 but went on to show that ELA-11 protect kidney cells from DNA damage-induced apoptosis and acute kidney injury (Chen et al., 2017). More recently, another study suggested that ELA-11 potently inhibited tumour growth in models of renal cancer (Soulet et al., 2020). Therefore, while further studies would be required to determine which of the longer isoforms are produced endogenously and quantify levels, this study provided the first evidence for the presence of endogenous ELA-11 in human tissues.

7.2.2 ACE2 Metabolise Apelin in Humans

It is well established that like many other peptides, apelin isoforms have a very short half-life. Therefore, due to the emerging critical therapeutic value of the apelin system in various disease states, a lot of effort has been put into understanding the degradation of the peptide and identification of potential proteolytic enzymes. This is required to devise methods of either inhibiting the proteolytic enzyme(s) or synthesising apelin analogues with greater plasma stability. These studies have led to the identification of specific enzymes involved in the degradation of apelin isoforms, which include; PCSK3 (Shin et al., 2013), plasma kallikrein (Fischer et al., 2019; Wang et al., 2019), ACE2 (Murza et al., 2014; Vickers et al., 2002; Wang et al., 2016; Yang et al., 2017c) and neprilysin (McKinnie et al., 2016). However, neprilysin is the only enzyme that can completely inactivate the apelin peptides by proteolytically cleaving the N-terminal RPRL motif required for binding to the apelin receptor (McKinnie et al., 2016). Hence, it is plausible that one of the mechanisms of cardioprotection mediated by neprilysin inhibitors or combined angiotensin-neprilysin inhibition in heart failure.
(Velazquez et al., 2019), may at least partly involve stabilisation of endogenous apelin peptides and thus, increased apelin activity.

ACE2 is well known to proteolytically remove the C-terminal phenylalanine residue from apelin peptides, including apelin-36, apelin-17 and [Pyr\(^1\)]apelin-13 to produce apelin-36\(_{1-35}\), apelin-17\(_{1-16}\) and [Pyr\(^1\)]apelin-13\(_{1-12}\) respectively (Vickers et al., 2002; Wang et al., 2016; Yang et al., 2017c). However, it was initially thought that ACE2 metabolites lacked the ability to bind and activate the apelin receptor. Recently, Yang et al., (2017c) demonstrated that it retained biological function at the apelin receptor that was similar to that of [Pyr\(^1\)]apelin-13. Given that these previous studies on apelin degradation were conducted either \textit{in vitro} or in rodents, which has a different repertoire of proteolytic enzymes to humans (Onorato et al., 2019), it remained unclear how [Pyr\(^1\)]apelin-13 was metabolised \textit{in vivo} in humans and what isoforms could be generated. This thesis provided further evidence on the interaction of ACE2 and [Pyr\(^1\)]apelin-13 in humans by demonstrating that following infusion of the peptide into healthy volunteers, the ACE2 metabolite, [Pyr\(^1\)]apelin-13\(_{1-12}\) was the most abundant metabolite generated. This is the first study where apelin was infused in humans, and potential cleavage sites or metabolites were identified. Additionally, other metabolites [Pyr\(^1\)]apelin-13\(_{1-10}\) and [Pyr\(^1\)]apelin-13\(_{1-6}\) were produced abundantly. However, it is not clear which enzyme is responsible for the generation of these latter metabolites and whether or not they are biologically active. In addition, the neprilysin metabolite, [Pyr\(^1\)]apelin-13\(_{1-5}\) ([Pyr\(^1\)]apelin-13\(_{6-13}\)) and [Pyr\(^1\)]apelin-13\(_{4-13}\) were identified, suggesting that neprilysin could equally cleave and inactivate endogenous apelin isoforms \textit{in vivo} in humans.

The metabolism of apelin by ACE2 in humans may have important implications for the treatment of some viral infections. ACE2 act as a receptor for beta-coronaviruses, including HCoV-NL63, SARS-CoV-1 and SARS-CoV-2 responsible for the current global pandemic (Hoffmann et al., 2020). This raised the possibility that exogenous apelin may have the therapeutic benefit of competing with the virus for receptor binding and thereby inhibit host entry and disease. A similar role has been described for apelin
in offering protection against HIV-1 infection, where the virus uses the apelin receptor as a co-receptor for host entry and infection (Zhou et al., 2003; Zou et al., 2000). Additionally, cleavage of membrane-bound ACE2 by ADAM17 results in the generation of soluble ACE2, which can still bind the virus but cannot internalise into host cells. This may serve to mop off viral particles and protect against Covid19 disease (Monteil et al., 2020). Therefore, given that apelin is known to upregulate ACE2, apelin may also offer protection against viral infections like Covid19 by promoting ACE2 shedding. Thus, further investigation in the apelin system, especially in Covid19 patients, may prove extremely important possibly as a potential therapy.

Furthermore, the methods developed in this thesis was capable of detecting endogenous circulating [Pyr]\_1\_apelin-13 (Nyimanu et al., 2019b). Previous attempts to detect endogenous circulating [Pyr]\_1\_apelin-13 by mass spectrometry involved solid-phase extraction followed by peptide enrichment (concentration) using cation exchange beads (Mesmin et al., 2010; Zhen et al., 2013); which is not only technically demanding but also time-consuming. However, this study utilised solid-phase extraction to detect endogenous [Pyr]\_1\_apelin-13 in human plasma. This is not only very easy to perform but also requires relatively shorter sample processing time, thus, representing a significant improvement in the measurement of apelin peptides by mass spectrometry.

### 7.3 Novel Apelin Receptor Analogues

The final aim of this thesis was to determine whether apelin analogues developed to have longer plasma half-lives could bind and activate the apelin receptor. Using various pharmacological assays, I have shown that apelin-36-[L28A] and apelin-36-[L28C(30kDa-PEG)] are biased ligands of the apelin receptor although the possibility of partial agonism cannot be excluded. Both ligands bound the apelin receptor with subnanomolar affinities in human and rat heart and demonstrated stronger potency in inhibiting cAMP accumulation over β-arrestin recruitment, consistent with a biased agonist. Apelin-36[L28A] and apelin-36 [L28C(30kDa-PEG)] were initially purported to
be 100- and 10,000-fold less potent at the apelin receptor compared to apelin-36. Still, these compounds significantly reduced blood glucose and improved glucose tolerance in diet-induced obese mice without having any effect on blood pressure (Galon-Tilleman et al., 2017). Therefore, this study demonstrated that the reported beneficial metabolic roles of apelin-36[L28A] and apelin-36 [L28C(30kDa-PEG)] were mediated by the apelin receptor possibly via recruitment of \( \text{G}_\alpha_i \) and \( \text{G}_\alpha_q \) signalling pathways resulting in the activation of AMPK, previously shown to mediate the metabolic roles of apelin (Bertrand et al., 2015).

In conclusion, this thesis demonstrated that ELA was expressed in other tissues and not only restricted to the kidney as previously thought in both humans and mice. This study has, for the first time, characterised the localisation of the apelin system in the human nephron, providing evidence for the expression of the apelin receptor protein in the juxtaglomerular apparatus. The clinical utility of these findings was explored in samples obtained from CKD patients. Thus, this study supports the therapeutic potential of targeting the apelin pathway in cardiorenal diseases. The final parts of this thesis explored the metabolism of ELA (in vitro) and apelin (in vivo) in humans and identified the metabolites of both peptides. This has provided useful information required to develop novel apelin and ELA analogues for experimental medicine. Evidence for the potential benefit of such analogues was also described.

### 7.4 Future Work

Data from chapter 3 of this thesis has been successfully used to obtain a grant for a clinical trial (NCT03956576) to investigate the possibility that apelin could be used to treat or reduce the severity of chronic kidney disease in patients. This study has begun, and the results appear promising so far. However, the role of apelin in the regulation of tubular function is unclear. Given the expression of the apelin pathway proteins from the glomerulus to collecting duct, it is likely to play a pivotal role in the regulation of tubular function. Moreover, apelin receptor colocalised with SGLT2 expression in the proximal convoluted tubule. SGLT2 in the proximal tubule is responsible for 90% of
glucose transport in this segment of the nephron and has recently been approved by FDA for treatment of diabetes and diabetic nephropathy (Gallo et al., 2015; Neuen et al., 2020).

Apelin has been suggested to reduce blood glucose level by increasing glucose utilisation in tissues like the skeletal muscle (Dray et al., 2008). Mechanistically, apelin was involved in a feedback loop with glucose in the intestine whereby increased glucose ingestion stimulated apelin secretion. The secreted apelin directly activated AMPK-mediated increase in the localisation of GLUT2 but decreased SGLT1 expression on enterocytes resulting in trans-epithelial glucose transport into the bloodstream (Dray et al., 2013). Thus, considering that similar mechanisms are involved in renal glucose handling, it is likely that apelin may play a role in the regulation of glucose homeostasis at the renal level possibly by modulating the activity of SGLT2 and this could be the focus of subsequent studies.

Additionally, this study showed for the first time the expression of the apelin receptor in the juxtaglomerular apparatus (JGA) and its colocalisation with NKCC2 in the thick ascending limb of the Loop of Henle. The JGA consists of the tubular (macula densa in TAL), and the vascular (comprising extraglomerular mesangial cells, juxtaglomerular cells and endothelial cells) component. It plays an important role in the regulation of tubuloglomerular feedback and renin secretion, where increased sodium chloride concentration at the macula densa is sensed by NKCC2, resulting in decreased renin secretion and increased production of adenosine (Bachmann and Oberbaumer, 1998; Ollerstam and Persson, 2002). Adenosine binds to and activates its receptors on the afferent and efferent arterioles resulting in vasoconstriction, and consequently decrease GFR and renal blood flow (Ollerstam and Persson, 2002). Therefore, the expression of apelin receptors in the JGA and colocalisation with NKCC2 may suggest that the apelin system have a critical role in the regulation of tubuloglomerular feedback or systemic blood pressure. Hence, it would be usefully for further studies such as a micropuncture experiment on this segment of the nephron to assess effects of apelin receptor agonism. Studies investigating the effect of apelin
infusion in animal models or humans on renin and angiotensin II levels may also advance understanding of the renal apelin the system.

In chapter 4 of this thesis, several shorter isoforms of the novel apelin receptor ligand, ELA, was identified including ELA-11, ELA-16, ELA-19 and ELA-20, which are likely to retain biological activity at the receptor. Therefore, further studies are required to investigate the pharmacology of these isoforms in vitro in cell-based assays and in vivo in animal models to assess their haemodynamic profile. Initial studies on the structure-activity relationship of ELA peptides demonstrated that ELA-16 retained nanomolar potencies similar to the longer ELA-32 peptide (Murza et al., 2016). Given that ELA-16 contain a disulphide bridge in its N-terminal, it is likely to be very stable in plasma and may prove important in experimental medicine studies. In support of this hypothesis, a recent study aimed at improving the binding affinity and plasma stability of ELA-11 led to the discovery of ELA-11 analogues with superior pharmacological properties (Wang et al., 2020). It went on to show that these analogues had better renoprotective effects in models of acute kidney injury, further confirming the potential therapeutic benefit of apelin receptor agonism in cardiorenal disease (Wang et al., 2020).

Additionally, the short fragments identified, ELA-8 and ELA-9, retained the critical amino acid residues required for binding previously described by Murza et al., (2016). Therefore, further studies could investigate the pharmacology of these fragments to determine whether they are agonists or antagonists at the apelin receptor. Such experiments may prove particularly crucial if they turn out to be antagonists since suitable apelin receptor antagonists are lacking (Read et al., 2019). Moreover, the enzymes producing the shorter isoforms identified in this study remains unknown and may also require further investigation.
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Appendix 1A. Chromatogram showing ELA-32(1-12) (A) and its C-terminal fragment ELA-20 (B). ELA-32(1-12) is unlikely to be able to bind the apelin receptor because the critical pharmacophores required for binding are in the C-terminus (ELA-20).
Appendix 1B. Chromatogram showing ELA-32\textsubscript{(1-13)} (A) and its C-terminal fragment ELA-19 (B). ELA-32\textsubscript{(1-13)} is unlikely to be able to bind the apelin receptor because the critical pharmacophores required for binding are in the C-terminus (ELA-19).
Appendix 1C. Chromatogram showing ELA-32(1-14) (A) and its C-terminal fragment ELA-18 (B). ELA-32(1-14) is unlikely to be able to bind the apelin receptor because the critical pharmacophores required for binding are in the C-terminus (ELA-18). This ELA-18 was oxidised on the methionine residue.
Appendix 1D. Chromatogram showing ELA-32 (B) and its C-terminal fragment ELA-17 (B). ELA-32 shown is the dioxidised form where both methionine residues are oxidised. ELA-17 is also oxidised on its methionine residue.
Appendix 1E. Chromatogram showing ELA-23 (A) and ELA-22 (B). ELA-23 was alkylated on its lysine residue shown in red while ELA-22 was oxidised on the methionine residue. Both of these isoforms are likely to be functional.
Appendix 1F. Chromatogram showing ELA-16_{1-14} (A) and ELA-19_{1-19} (B) derived from des-Pro^{17\_\_}ELA-19 and des-Pro^{16\_\_}ELA-16 respectively. These fragments may have been generated by ACE2 activity.
Appendix 1G. Chromatogram showing ELA-8 (A) and ELA-9 (B). It is not clear whether these fragments would bind the apelin receptor although they contain the critical pharmacophores required for binding.
Appendix 1H. Protein network showing all the proteins identified in the coronary artery using the STRING database.
Appendix 1. Protein network showing all the proteins identified in the rat kidney using the STRING database.