frontiers

1 **The G Protein Biased Small Molecule Apelin Agonist CMF-019 is Disease Modifying in**
2 **Endothelial Cell Apoptosis *In vitro* and Induces Vasodilatation without Desensitisation *In vivo***

3 **Running Title: Disease modifying effects of CMF-019**

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16 **Keywords:** apelin, apoptosis, bias, cardiovascular, G protein-coupled receptor, *in vivo*, pulmonary
17 artery endothelial cell.

18 **Abstract**

19 Signaling through the apelin receptor is beneficial for a number of diseases including pulmonary
20 arterial hypertension. The endogenous small peptides, apelin and elabela/toddler, are downregulated
21 in pulmonary arterial hypertension but are not suitable for exogenous administration owing to a lack
22 of bioavailability, proteolytic instability and susceptibility to renal clearance. CMF-019, a small
23 molecule apelin agonist that displays strong bias towards G protein signaling over β-arrestin (~400
24 fold), may be more suitable.

25 This study demonstrates that in addition to being a positive inotrope, CMF-019 caused dose-
26 dependent vasodilatation *in vivo* (50nmol 4.16±1.18mmHg, $^{**}p<0.01$; 500nmol 6.62±1.85mmHg,
27 $^{**}p<0.01$), without receptor desensitization. Furthermore, CMF-019 rescues human pulmonary artery
28 endothelial cells from apoptosis induced by tumor necrosis factor α and cycloheximide (5.66±0.97%,
29 $^{**}p<0.01$) by approximately 50% of that observable with rhVEGF (11.59±1.85%, $^{**}p<0.01$),
30 suggesting it has disease-modifying potential *in vitro*.

31 CMF-019 displays remarkable bias at the apelin receptor for a small molecule and importantly
32 recapitulates all aspects of the cardiovascular responses to the endogenous ligand, [Pyr[1]]apelin-13, *in*
33 *vivo*. Additionally, it is able to protect human pulmonary artery endothelial cells from apoptosis,
34 suggesting that the beneficial effects observed with apelin agonists extend beyond hemodynamic
35 alleviation and address disease etiology itself. These findings support CMF-019 as a G protein-biased

36 small molecule apelin agonist *in vitro* and *in vivo* that could form the basis for the design of novel
37 therapeutic agents in chronic diseases, such as, pulmonary arterial hypertension.

38 # 1 Introduction

39 The apelin receptor, a class A G protein coupled receptor (O'Dowd et al., 1993) has two endogenous
40 peptide ligands, apelin (Tatemoto et al., 1998) and elabela/toddler (ELA) (Chng et al., 2013; Pauli et
41 al., 2014) The apelin system is a therapeutic target in diseases (Read et al., 2019), such as diabetes
42 (Castan-Laurell et al., 2012), fibrosis (Huang et al., 2016), heart failure (Berry et al., 2004; Jia et al.,
43 2006; Alturi et al., 2007; Koguchi et al., 2012; Pang et al., 2014;) and pulmonary arterial
44 hypertension (PAH) (Falcão-Pires et al., 2009; Yang et al., 2015). In PAH, apelin (Goetze et al.,
45 2006; Alastalo et al., 2011; Kim et al., 2013) and ELA (Yang et al., 2017) are downregulated but
46 receptor expression is maintained (Andersen et al., 2009; Falcão-Pires et al., 2009) therefore
47 replacing the missing ligands could be a therapeutic strategy. In a model of PAH [Pyr^1]apelin-13
48 (Falcão-Pires et al., 2009; Maguire et al., 2009) and ELA-32 (Yang et al., 2017) prevented disease
49 onset, however, both lack oral bioavailability and are susceptible to proteolytic cleavage and renal
50 excretion. Furthermore, repeated agonist stimulation leads to β-arrestin recruitment and receptor
51 internalization, potentially blunting therapeutic efficacy.

52 A preferable strategy is to identify G protein biased apelin ligands (Brame et al., 2015). We have
53 identified a small molecule, CMF-019 (Hachtel, et al., 2014), that is a positive inotrope *in vivo* and
54 possesses strong G protein bias. CMF-019 has nanomolar affinity for the apelin receptor in both
55 human and rat heart and whereas this compound inhibits $G\alpha_i$ mediated cAMP accumulation with
56 sub-nanomolar potency comparable to [Pyr^1]apelin-13 it is over two orders of magnitude less
57 efficient in recruiting β-arrestin or inducing apelin receptor internalization compared to the
58 endogenous agonist (Read et al., 2016). In this study we have investigated whether CMF-019 alters
59 apoptosis in human pulmonary arterial endothelial cells (PAECs), a driver of early disease phase
60 (Wilson et al., 1992; Rabinovitch 2012). We aimed to confirm that a G protein biased compound
61 produces vasodilatation *in vivo*, (the main mechanism of action for most current PAH therapies)
62 without desensitization, as recent studies have suggested that apelin-mediated vasodilatation may
63 occur via β-arrestin signaling (El Messari et al., 2004; Iturrioz et al., 2010; Ceraudo et al., 2014).

64 # 2 Materials and Methods

65 ## 2.1 Materials

66 Chemicals were obtained from Sigma Aldrich Co. Ltd (Poole, UK) unless otherwise stated.
67 [Pyr^1]apelin-13 (purity >98%) was from Severn Biotech (Kidderminster, UK). CMF-019 was
68 synthesized as a potassium salt (Read et al., 2016), initially in the School of Chemistry, University of
69 Leeds (purity>95%) and later by Tocris (purity 99.3%) (Bristol, UK). All animal care and rodent
70 experiments complied with the Home Office (UK) guidelines under the Animals (Scientific
71 Procedures) Act 1986 Amendment Regulations (SI 2012/3039) and were approved by the local ethics
72 committee (University of Cambridge Animal Welfare and Ethical Review Body).

73 ## 2.2 Rescue of Human Pulmonary Artery Endothelial Cell Apoptosis

74 The effects of CMF-019 on endothelial cell apoptosis were tested and compared with recombinant
75 human vascular endothelial growth factor (rhVEGF; R&D Systems, Minneapolis, MN, USA) using
76 human PAECs (Lonza; Cambridge, UK; n=5 donors: 1(lot#0000479486), 2(lot#4F3041),
77 3(lot#4F3034), 4(lot#000657513) and 5(lot#0000662151), passages 4-6) as previously described (28

78 Long et al., 2015) following protocol optimization. Briefly, PAECs were seeded in six-well tissue
79 culture plates at 200,000cells/well in endothelial growth medium-2 (EGM-2; Lonza, PromoCell; UK)
80 with 10% fetal bovine serum (FBS, Gibco™, NY, USA) and allowed to attach. On the next day,
81 wells were washed with PBS and the media changed to either endothelial basal medium 2 (EBM-2;
82 Lonza, PromoCell) with 2% FBS or 10% FBS controls. CMF-019 (1-10μM) or rhVEGF (10ng/mL)
83 were added to the wells and incubated for 18 hours. Apoptosis was induced by incubating the cells
84 with tumor necrosis factor α (TNFα; R&D Systems, 1.5ng/mL) and cycloheximide (CHX; 20μg/ml)
85 for 5 hours in the experimental wells. Control wells did not receive TNFα/CHX treatment. Cells were
86 then washed in PBS, trypsinized (Lonza), transferred into 1x binding buffer for the apoptosis assay
87 (Thermoscientific; Waltham, MA, USA) and stained with anti-annexin FITC-conjugated antibody
88 (1:2 stock dilution) and propidium iodide (PI, 20μg/mL) for 15 minutes at room temperature. Cells
89 were filtered through 50μM filters (Sysmex/Partec; Görlitz, Germany) and kept on ice before flow
90 cytometry (Canto II, BD Biosciences; San Jose, CA, USA). For each condition 10,000 events were
91 recorded. Data analysis was performed on FlowJo v10 (FlowJo LLC; Ashland, OR, USA).
92 $Annexin^+/PI^+$ cells were classified as 'dead', $Annexin^+/PI^-$ cells 'apoptotic' and $Annexin^-/PI^-$ as
93 'healthy'. Gates were adjusted such that approximately equal numbers of 'healthy' and 'apoptotic'
94 cells occurred in the TNFα/CHX treated group as this provided a large window for either further
95 induction or rescue of apoptosis. The raw percentage of cells in each gate were used in the data
96 analysis and some variability in the basal amount of apoptotic induction between replicates was
97 observed. A matched ANOVA was utilized to remove this variability and compare data trends.

98 2.3 *In vivo* Catheterization to Assess Cardiovascular Responses to CMF-019 in Normotensive 99 Male Sprague-Dawley Rats

100 Normotensive male Sprague-Dawley rats (271±3g, n=17) underwent left ventricular and femoral
101 artery catheterization to assess cardiac and vascular changes upon bolus CMF-019 administration.
102 Left ventricular catheterization was performed as previously described (Pacher et al., 2008; Read et
103 al., 2016; Yang et al., 2017) and the femoral catheterization protocol was developed as an extension
104 to this protocol. In brief, rats were anaesthetized with gaseous isoflurane (3-2.5% for initial
105 induction, maintenance and surgery, and 1.5% for hemodynamics measurement; 1.5L/min oxygen).
106 The right external jugular vein was exposed, cannulated and flushed with heparin solution (2%) made
107 up in saline (0.9% saline, pH5, Macopharma; Tourcoing, FR). The right common carotid artery was
108 then located and a catheter (SPR-869, Millar Inc.; Houston, TX, USA) inserted and advanced to the
109 left ventricle. Once a stable pressure-volume loop could be observed, the femoral artery was exposed
110 and a second identical catheter inserted to record arterial pressure responses simultaneously to the
111 ventricular responses. Three successive doses of either CMF-019 (50-5000nmol, 0.5mL, 0.9% saline,
112 pH9), $[Pyr^1]$apelin-13 (10-150nmol, 0.5mL, 0.9% saline, pH5) or saline controls were then
113 administered intravenously via the jugular vein catheter, followed by a saline flush (0.9%, 0.1mL,
114 pH5) at a minimum of ten minute intervals or when a stable baseline was reached before the next
115 injection. All animals received a 50nmol dose of $[Pyr^1]$apelin-13 as a fourth dose after they had
116 received their first three doses whether they be saline, CMF-019 or $[Pyr^1]$apelin-13. The dosing
117 schedule is summarized in figure 1. Data were acquired using the MPVS Ultra system
118 (ADIstruments; Dunedin, NZ) and analyzed using LabChart 8 (ADIstruments). Values for the
119 maximal change in arterial pressure, left ventricular systolic pressure (LVSP), stroke volume, cardiac
120 output, heart rate, contractility (dP/dt_{MAX}) and lusitropy (dP/dt_{MIN}) from baseline were calculated
121 from the raw data and compared. Following completion of the measurements the animal was
122 euthanized by exsanguination under high flow isoflurane (5%).

123 **2.4 Statistical Analysis**

124 All data are expressed as mean±SEM values and statistical analyses were performed with GraphPad
125 Prism 6 (La Jolla, CA, USA) unless otherwise stated. For rescue of human PAEC apoptosis,
126 experiments were performed at least in triplicate. Donors 4 and 5 were excluded from the analysis as
127 they showed a particularly small window of apoptotic cell induction using TNF-α and CHX
128 (2.80±1.60%), this was not significantly different to the EBM-2 2% FBS control (Matched ANOVA).
129 Of the remaining donors, technical replicates were excluded if the apoptotic induction was less than
130 5%. The average induction of apoptosis of the analyzed experiments was 19.53±1.76%. Data were
131 normally distributed using a D'Agostino-Pearson omnibus K^2 test and trends were compared using a
132 matched ANOVA to account for variability observed in the basal amount of apoptotic induction
133 observed between replicates. For the acute *in vivo* studies, normality has been confirmed by
134 analyzing data collected over a number of experiments using the D'Agostino-Pearson omnibus K^2
135 test. Consequently, cardiovascular parameters measured in saline were compared to CMF-019 and
136 [Pyr^1]apelin-13 treated animals using a two-tailed Student's t-test. Statistical significance was taken
137 as 5%.

138 **3 Results**

139 **3.1 CMF-019 Rescued Human PAEC Apoptosis Induced by TNFα and CHX**

140 The ability of CMF-019 to prevent TNFα/CHX induced apoptosis in human PAECs was tested at 1
141 and 10μM (figure 2 and figure 3). TNFα/CHX significantly increased the percentage of annexin$^+$/PI$^-$
142 cells (19.54±1.76%, $^{####}p<0.0001$) compared to the EBM-2 2% FBS control and rhVEGF was able to
143 partially rescue this (11.59±1.85%, $^{**}p<0.01$), as was CMF-019 at 1μM (5.66±0.97%, $^{**}p<0.01$).
144 CMF-019 at 10μM displayed no significant rescue despite trending towards significance
145 (4.38±1.48%, ns) (figure 3).

146 In control experiments assessing apoptotic responses and rescue to growth factor and serum
147 starvation (figure 4), growth factor and serum starvation significantly increased the percentage of
148 annexin$^+$/PI$^-$ cells (9.33±1.94%, $^{##}p<0.01$) compared to the EGM-2 10% FBS 'healthy' control and
149 rhVEGF was able to completely rescue this (8.86±1.07%, $^{***}p<0.001$). However, CMF-019 at 1 μM
150 (1.04±0.86%, ns) and 10μM (-0.91±0.86%, ns) displayed no rescue in these conditions.

151 **3.2 CMF-019, a G Protein-Biased Small Molecule, Reduced Peripheral Artery Pressure *In***
152 ***Vivo***

153 Measured by catheterization of the femoral artery, bolus CMF-019 administration via the jugular vein
154 revealed reproducible peripheral reduction in femoral artery pressure compared to saline at 50nmol
155 (4.16±1.18mmHg, $^{**}p<0.01$) and 500nmol (6.62±1.85mmHg, $^{**}p<0.01$) (figure 5A). At the highest
156 dose of 5000nmol the response was not significant. [Pyr^1]apelin-13 produced a larger dose dependent
157 decrease in blood pressure at all doses administered (10nmol 12.06±3.51mmHg, $^{*}p<0.05$; 50nmol
158 31.24±8.70mmHg, $^{**}p<0.01$ and 150nmol 31.75±9.53mmHg, $^{*}p<0.05$) (figure 5B).

159 **3.3 Cardiac Responses *In Vivo* to CMF-019 and [Pyr^1]Apelin-13**

160 CMF-019 increased cardiac contractility (500nmol 251±89mmHg/s, $^{*}p<0.05$), stroke volume
161 (50nmol 2.63±0.82RVU, $^{**}p<0.01$; 500nmol 2.48±0.87RVU, $^{*}p<0.05$), cardiac output (50nmol
162 1097±284RVU/min, $^{**}p<0.01$; 500nmol 1012±340RVU/min, $^{*}p<0.05$) and produced a small
163 elevation in heart rate (500nmol 5.46±2.32BPM, $^{*}p<0.05$) (figure 6). CMF-019 decreased LVSP

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(50nmol 1.88±0.57mmHg, **p<0.01; 500nmol 2.23±0.80mmHg, *p<0.05) in concert with the arterial pressure. Avery small decrease in lusitropy was also observed (50nmol 210±70mmHg/s, *p<0.05). Similar to CMF-019, Pyr^{1}]apelin-13 increased cardiac contractility (150nmol 1920±178mmHg/s, ***p<0.001), stroke volume (10nmol 4.72±0.98RVU, **p<0.01; 50nmol 9.70±1.93RVU, **p<0.01; 150nmol 9.84±3.02RVU, *p<0.05), cardiac output (10nmol 2008±464RVU/min, **p<0.01; 50nmol 3618±818RVU/min, **p<0.01; 150nmol 3961±1154RVU/min, *p<0.05) and decreased LVSP (10nmol 5.66±0.81mmHg, ***p<0.001; 50nmol 11.02±1.70mmHg, ***p<0.001; 150nmol 9.47±1.16mmHg, ****p<0.0001). [Pyr^{1}]apelin-13 produced a small elevation in heart rate (150nmol 12.94±4.95BPM, *p<0.05) and a small decrease in lusitropy was observed (150nmol 1143±378mmHg/s, *p<0.05) which was consistent with CMF-019 (figure 6). The time course for the effect of both compounds was similar. No adverse effects were observed at any of the doses administrated.

3.4 CMF-019 Did Not Desensitize the Apelin Receptor *In Vivo*

To study desensitization at the apelin receptor *in vivo*, a fourth dose of [Pyr^{1}]apelin-13 at 50nmol was administered to the saline, CMF-19 and [Pyr^{1}]apelin-13 treatment groups (figure 7). The x-axis denotes whether the preceding three doses were either saline or increasing doses of CMF-019 or [Pyr^{1}]apelin-13. The responses to [Pyr^{1}]apelin-13 in CMF-019 treated animals compared to saline treated animals were not significantly different for any parameter indicating that CMF-019 did not significantly desensitize the receptor compared to saline. In contrast there was a trend for the response to 50nM [Pyr^{1}]apelin-13 to be blunted in for all parameters following the three successive [Pyr^{1}]apelin-13 doses that reached significance for the contractility response (P<0.001) consistent with desensitization.

4 Discussion

4.1 CMF-019 Modified Disease in a Human PAEC Apoptotic Model

In the advanced stages of PAH, pathological remodeling of pulmonary vessels occurs including endothelial proliferation and the development of distinctive plexiform lesions which may in part be driven by imbalances in apelin signaling (Andersen et al., 2011; Yang et al., 2015). However, in early disease it is thought that endothelial cell apoptosis, leading to vascular dysfunction may drive onset (Wilson et al., 1992; Sakao et al., 2005; Rabinovitch, 2012). Apelin has been suggested to mitigate these effects and promotes survival of pulmonary vascular endothelial cells (Alastalo et al., 2011; Kim et al., 2013). Here we have studied the ability of the G protein-biased small molecule apelin agonist, CMF-019, to promote human PAEC survival in response to apoptotic stimulation with TNFα/CHX. TNFα may prevent apoptosis of endothelial cells through activation of the NF-κB pathway, however, in conditions of global protein synthesis suppression, such as, with concurrent application of CHX, signaling through TNF-R1 dominates leading to JNK phosphorylation and induction of apoptosis (Wajant et al., 2003).

Both CMF-019 and rhVEGF rescued endothelial cells from TNFα/CHX induced apoptosis. The rescue with CMF-019 was approximately 50% of that observed for the positive control, rhVEGF, and supports a role for apelin agonists in preventing endothelial damage in healthy human PAECs when challenged with an apoptotic stimulus. The mechanism by which this occurs is not well characterized, however, both [Pyr^{1}]apelin-13 and ELA-32, endogenous agonists of the apelin receptor (Read et al., 2019), promote ERK1/2 phosphorylation in human PAECs (Yang et al., 2017), while some apelin isoforms have been shown to downregulate the JNK and p-38 pathways in osteoblasts (Tang et al., 2007) and neurons (Liu et al., 2018). These pathways both have known roles

208 in apoptosis. Furthermore, there is evidence that apelin regulates myocyte enhancer factor 2, which in
209 turn can activate miR-424/miR-503 and genes contributing to endothelial cell homeostasis. This axis
210 may also be beneficial in PAH by acting on pulmonary arterial smooth muscle cells. Here it inhibits
211 the expression of fibroblast growth factor 2 and its receptor, and thus, exerts anti-proliferative effects,
212 potentially preventing their over-proliferation and subsequent vessel muscularization (Alastalo et al.,
213 2011; Kim et al., 2013; Helenius et al., 2015; Kim et al., 2015). Finally, agents that activate apelin
214 expression or act as downstream effectors of apelin signaling have demonstrated beneficial effects in
215 PAH animal models (Kim et al., 2013; Spiekerkoetter et al., 2013; Bertero et al., 2014; Nickel et al.,
216 2015).

217 Interestingly, CMF-019 did not rescue apoptosis induced by serum and growth factor starvation in
218 the control arm of the experiment, although the positive control rhVEGF did. This suggests that
219 rescue from serum and growth factor starvation is through a different mechanism to rescue from
220 TNFα/CHX induced apoptosis. Growth factor and serum starvation has been widely observed in
221 various endothelial cell lines (Karsan et al., 1997; Gerber et al., 1998b; Raymond et al., 2004; Date et
222 al., 2005; Ueda et al., 2005; Gama Sosa et al., 2016) and the mechanisms for rescue with rhVEGF are
223 thought to occur by both upregulation of the MAPK/ERK pathway alongside downregulation of JNK
224 pathway (Gupta et al., 1999), leading to enhanced bcl-2 and decreased bax signaling (Karsan et al.,
225 1997; Gerber et al., 1998a; Ueda et al., 2005). In contrast, TNFα/CHX stimulates apoptosis primarily
226 through the TNF-R1 leading to induction of the JNK and p-38 MAPK pathways. On balance there is
227 greater evidence of apelin promoting ERK/MAPK signaling, especially in PAECs, and perhaps
228 activation of this pathway was more efficacious in rescuing from JNK/p-38 mediated apoptosis
229 induced by TNFα/CHX, rather than the multiple pathway mechanisms of apoptosis induced by serum
230 and GF starvation.

231 This fact that CMF-019 rescued only in the TNFα/CHX condition may suggest it as a potential
232 disease modifier in that it prevented endothelial cell apoptosis in conditions of a severe stimulus but
233 did not have an effect in response to the weaker serum and GF starvation stimulus. Similar results
234 were observed with the cyclic peptide, MM07, also a G protein biased apelin agonist which was
235 effective in preventing PAH onset in a rat MCT model, suggesting a potential mechanism for
236 modification of disease onset (Yang et al., 2019). Although [Pyr[1]]apelin-13 was administered in
237 similar cell apoptosis experiments (data not shown), no rescue was observed and this was thought to
238 be due either to proteolytic breakdown or high plasma protein binding in serum over the prolonged
239 18 hour incubation time. Protease inhibitors cannot be used in this assay to prevent apelin breakdown
240 as these would confound it by negating protein synthesis inhibition by CHX (unpublished
241 observation).

242 **4.2 CMF-019 Reduced Peripheral Artery Pressure and Increased Cardiac Output *In vivo***

243 CMF-019 administered by bolus intravenous injection through a jugular vein cannula in
244 normotensive male Sprague-Dawley rats induced both a reduction in pressure recorded in the femoral
245 artery and cardiac responses, as did [Pyr[1]]apelin-13. Consistent with cardiac responses that we have
246 previously reported to CMF-019 and [Pyr[1]]apelin-13 (Read et al., 2016), in this study both apelin
247 agonists displayed reproducible enhancement of contractility, stroke volume and cardiac output. The
248 maximum response to CMF-019 was smaller than that observed for [Pyr[1]]apelin-13 and this was
249 likely due to the limited concentrations of CMF-019 that could be attained *in vivo* as previously
250 stated. In our previous report CMF-019 had little effect on LVSP with a small increase seen at the
251 highest dose. We postulated that the lack of effect of CMF-019 may be explained either by limited
252 solubility or that vasodilatation resulting from apelin receptor activation may be a β-arrestin mediated

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253 response. We therefore extended the protocol in this study to also measure pressure changes in the
254 femoral artery. In the current study we observed a small decrease in LVSP with CMF-019 consistent
255 with the larger decreases obtained with [Pyr[1]]apelin-13. Crucially, CMF-019 induced a reduction in
256 femoral artery pressure following administration which has not been previously demonstrated. This
257 response most likely reflects a decrease in peripheral resistance, consistent with CMF-019, like
258 [Pyr[1]]apelin-13, acting as vasodilators, as both molecules increase stroke volume. It has been
259 suggested that longer apelin peptides, such as apelin-17, display β-arrestin bias by reaching deeper
260 within the apelin binding pocket and through these contacts they both internalize the receptor and
261 signal to produce vasodilatation (El Messari et al., 2004; Iturrioz et al., 2010; Ceraudo et al., 2014).
262 This theory for β-arrestin bias seems likely and we observe that smaller and cyclic peptides, such as
263 MM07, possess G protein bias, while the small molecule CMF-019 displays the greatest G protein
264 bias we have observed (Read et al., 2016). However, from our results this does not correlate with a
265 reduced ability to produce vasodilatation. In fact, given the marked bias of CMF-019 towards the G
266 protein pathway, it suggests that vasodilatation is possible without engagement of β-arrestin signaling
267 and receptor internalization. This is further supported by the sustained vasodilatation observed in
268 humans *in vivo* with MM07 (Brame et al., 2015).

269 4.3 CMF-019 Did Not Desensitize the Apelin Receptor *In Vivo*

270 It has previously been shown that CMF-019 displays weak activity in recruiting β-arrestin and
271 internalizing the apelin receptor *in vitro* (Read et al., 2016). Therefore, to assess the ability of CMF-
272 019 to internalize the apelin receptor *in vivo*, a protocol was devised whereby subsequent to the three
273 doses of saline or three increasing doses of CMF-019 or [Pyr[1]]apelin-13 a fourth dose comprising of
274 [Pyr[1]]apelin-13 50nmol was administered to all saline, CMF-19 and [Pyr[1]]apelin-13 treated animals.
275 These fourth doses were compared to assess whether there was any desensitization of the response as
276 a consequence of the previous doses of CMF-019 or [Pyr[1]]apelin-13 administered. Although this
277 study was limited by the number of animals that could be used and a high variance in the 50nmol
278 [Pyr[1]]apelin-13 responses, there is a clear trend across the parameters. There was very little
279 difference in responses to 50nmol [Pyr[1]]apelin-13 in any parameter when they had received saline or
280 CMF-019 for the first three doses. In contrast the increase in contractility produced by 50nM
281 [Pyr[1]]apelin-13 following saline was significantly attenuated following the three doses of
282 [Pyr[1]]apelin-13 and there was a trend for other parameters to be blunted. A limitation of this study is
283 that the maximum response induced by CMF-019 was lower than that to [Pyr[1]]apelin-13 however
284 overall the data suggested that CMF-019 did not desensitize the apelin receptor *in vivo*.

285 4.4 Conclusions

286 The identification of CMF-019, the first G protein biased small molecule apelin agonist, represents
287 an advance in the development of small molecule apelin agonists for use as experimental tool
288 compounds for *in vitro* and *in vivo* study. This is confirmed by the fact it is already widely
289 commercially available and methods to improve the synthesis of the molecule have been attempted
290 (Trifonov et al., 2018). Furthermore, it has potential as a starting point or stimulus for the
291 development of newer biased small molecule therapeutics at the apelin receptor with improved
292 pharmacokinetic profiles (Narayanan et al., 2020).

293 In this study, we have shown that CMF-019 is able to rescue endothelial cell apoptosis that has been
294 shown to be a driver of early PAH pathogenesis. Moreover, we have demonstrated that CMF-019 is
295 able to induce vasodilatation despite its pronounced G protein bias, in addition to cardiac inotropy.
296 Overall, this study supports further investigation of novel G protein biased apelin agonists such as
297 CMF-019 but with improved pharmacokinetics as potential therapeutics in PAH.

298 **Conflict of Interest**

299 *The authors declare that the research was conducted in the absence of any commercial or financial*
300 *relationships that could be construed as a potential conflict of interest.*

301 **Author Contributions**

302 *AD, JM and CR contributed to the conception and design of the study. Experimental work was*
303 *carried out by CR, DN, PY, TW RK and JM. Data analysis was performed by CR and JM. Apelin*
304 *agonists with possible functional bias were selected by RG and RF for synthesis based on literature*
305 *analysis and molecular modelling. Synthesis was performed by CF. Salt selection was based on*
306 *precedent to optimise solubility by RF, RG and CF. CR, JM and AD wrote the first draft of the*
307 *manuscript. Funding acquisition AD and JM. All authors read and approved the submitted version.*

308 **Funding**

309 This work was supported by the British Heart Foundation [FS/14/59/31282 to CR]; Wellcome Trust
310 [WT107715/Z/15/Z to AD, RK], Wellcome Trust Programme in Metabolic and Cardiovascular
311 Disease [096822/Z/11/Z to PY, 203814/Z/16/A to TW]; Medical Research Council [MRC
312 WT107715/Z/15/Z to APD, JJM, RCG] ; Pulmonary Hypertension Association UK; Cambridge
313 Biomedical Research Centre Biomedical Resources Grant University of Cambridge [099156/Z/12/Z];
314 Engineering and Physical Sciences Research Council [EP/M506552/1 to CMF]; and the Biomedical
315 Health Research Centre, University of Leeds [CMF, RF].

316 **Acknowledgments**

317 We thank the Papworth Hospital Research Tissue Bank and NIHR Cambridge Biomedical Research
318 Centre.

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478 **Figure Legends**

479 **Figure 1:** Dosing schedule for the second *in vivo* study. The left ventricle and femoral artery were
480 catheterized in normotensive male Sprague-Dawley rats. Subsequently, the animals were randomly
481 chosen to receive either three doses of saline, CMF-019 or [Pyr^1]apelin-13, before a fourth dose of
482 50nmol [Pyr^1]apelin-13 was administered regardless of the previous doses administered. Doses were
483 administered at ten minute intervals or when a stable baseline was reached after which the animal
484 was terminated by exsanguination under high flow isoflurane.

485 **Figure 2:** Representative contour plot flow data of human PAECs induced to apoptosis with
486 TNFα/CHX and rescued with either rhVEGF or CMF-019 at 1μM. Cells were incubated in EGM-2
487 with 10% FBS (control); EGM-2 with 2% FBS and TNFα/CHX (TNFα/CHX) treatment for 5hrs to
488 induce apoptosis before rhVEGF (10ng/ml) or CMF-019 (1-10μg/ml) was added for a further 18hrs.
489 PI staining is displayed on the x-axis and annexin-V FITC staining on the y-axis. The percentage of
490 cells in each quadrant are shown in the corners of the quadrant.

491 **Figure 3:** Dot plot of the percentage of annexin$^+$/PI$^-$ human PAECs in each experimental condition
492 with mean±SEM data superimposed. Cells were incubated in EGM-2 with 2% FBS alone (control) or
493 EGM-2 with 2% FBS and treated with TNFα/CHX (TNFα/CHX) for 5hrs to induce apoptosis before
494 rhVEGF (10ng/ml), or CMF-019 (1-10μg/ml) was added to TNFα/CHX treated cells for a
495 further18hrs. TNFα/CHX significantly increased the percentage of annexin$^+$/PI$^-$ human PAECs and
496 this could be rescued by 10ng/mL rhVEGF and CMF-019 at 1μM. Matched ANOVA comparing
497 each condition to TNFα/CHX **$p<0.01$. #### indicates $p<0.0001$ compared to control (EBM-2 2%
498 FBS).

499 **Figure 4:** Dot plot of the percentage of annexin$^+$/PI$^-$ human PAECs in each experimental condition
500 with mean±SEM data superimposed. Cells were incubated in EGM-2 with 10% FBS alone ('growth
501 factor control'), EGM-2 with 2% FBS alone (serum starvation) or EGM-2 with 2% FBS and 18hrs
502 treatment with rhVEGF (10ng/ml) or CMF-019 (1-10μg/ml). Growth factor and serum starvation
503 (EBM-2 2% FBS) significantly increased the percentage of annexin$^+$/PI$^-$ human PAECs and this
504 could be rescued by 10ng/mL rhVEGF. CMF-019 did not rescue. Matched ANOVA comparing each
505 condition to the EBM-2 2% FBS condition ***$p<0.001$. ## indicates $p<0.01$ compared to the
506 'healthy' control (EGM-2 10% FBS).

507 **Figure 5:** The arterial pressure change in response to CMF-019 and [Pyr^1]apelin-13 *in vivo*.
508 Decreases in arterial pressure in anaesthetized male Sprague-Dawley rats to (**A**) intravenous CMF-
509 019 potassium salt (■, n=8) and (**B**) [Pyr^1]apelin-13 (apelin, ▲, n=5) compared to saline (●, n=4)
510 control. Each dose was compared by a Student's t-test to its corresponding saline control as doses
511 were administered cumulatively (*$p<0.05$, **$p<0.01$).

512 **Figure 6:** Cardiovascular responses to CMF-019 and [Pyr^1]apelin-13 *in vivo*. Graphs showing
513 changes in contractility (dP/dt_{MAX}; **A-B**) left ventricular systolic pressure (LVSP; **C-D**), stroke
514 volume (SV; **E-F**), cardiac output (CO; **G-H**), heart rate (HR; **I-J**) and relaxation (dP/dt_{MIN}; **K-L**) for
515 CMF-019 potassium salt (■, n=8, **A, C, E, G, I, K**) and [Pyr^1]apelin-13 (apelin, ▲, n=5, **B, D, F, H,**
516 **J, L**) compared to saline (●, n=4, A-H) when injected intravenously into anaesthetized male
517 Sprague-Dawley rats. Each dose was compared by a Student's t-test to its corresponding saline
518 control as doses were administered cumulatively (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

519 **Figure 7:** Cardiovascular responses to [Pyr^1]apelin-13 at 50nmol administered as a fourth dose
520 following three successive doses of either saline pH9, CMF-019 or [Pyr^1]apelin-13 *in vivo*. Graphs

521 show changes in arterial pressure (**A**), contractility (dP/dt_{MAX}) (**B**), left ventricular systolic pressure
522 (LVSP; **C**), stroke volume (SV; **D**), cardiac output (CO; **E**), heart rate (HR; **F**) and relaxation
523 (dP/dt_{MIN}) (**G**) to 50nmol [Pyr^1]apelin-13 in anaesthetized male Sprague-Dawley rats previously
524 administered with three doses of either saline (●, n=4) or CMF-019 potassium salt (■, n=8) or
525 Pyr^1]apelin-13 (▲, n=5). The response to [Pyr^1]apelin-13 after CMF-019 or [Pyr^1]apelin-13 was
526 compared by a Student's t-test to that after saline control (***$p<0.001$).

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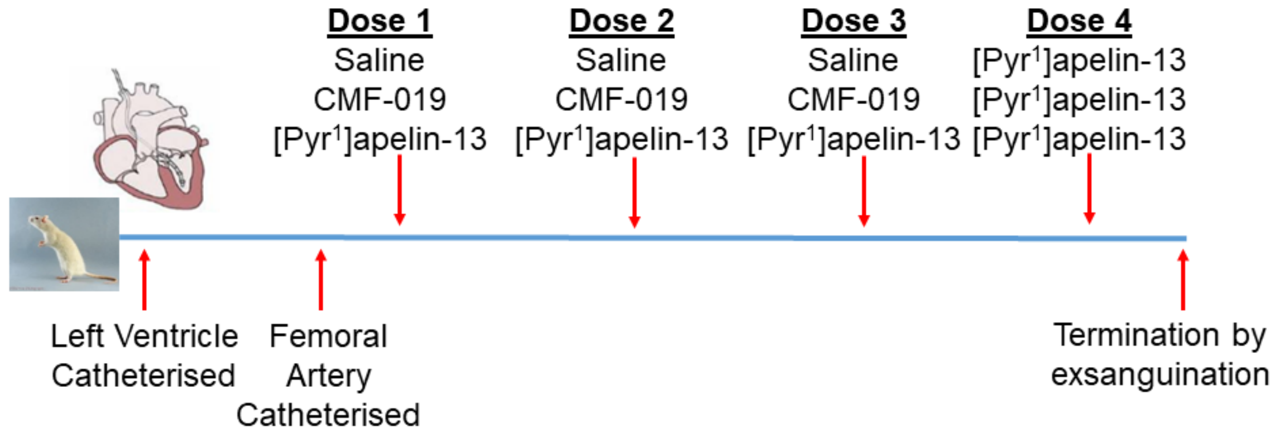
528 **Figures**

529 Figure 1



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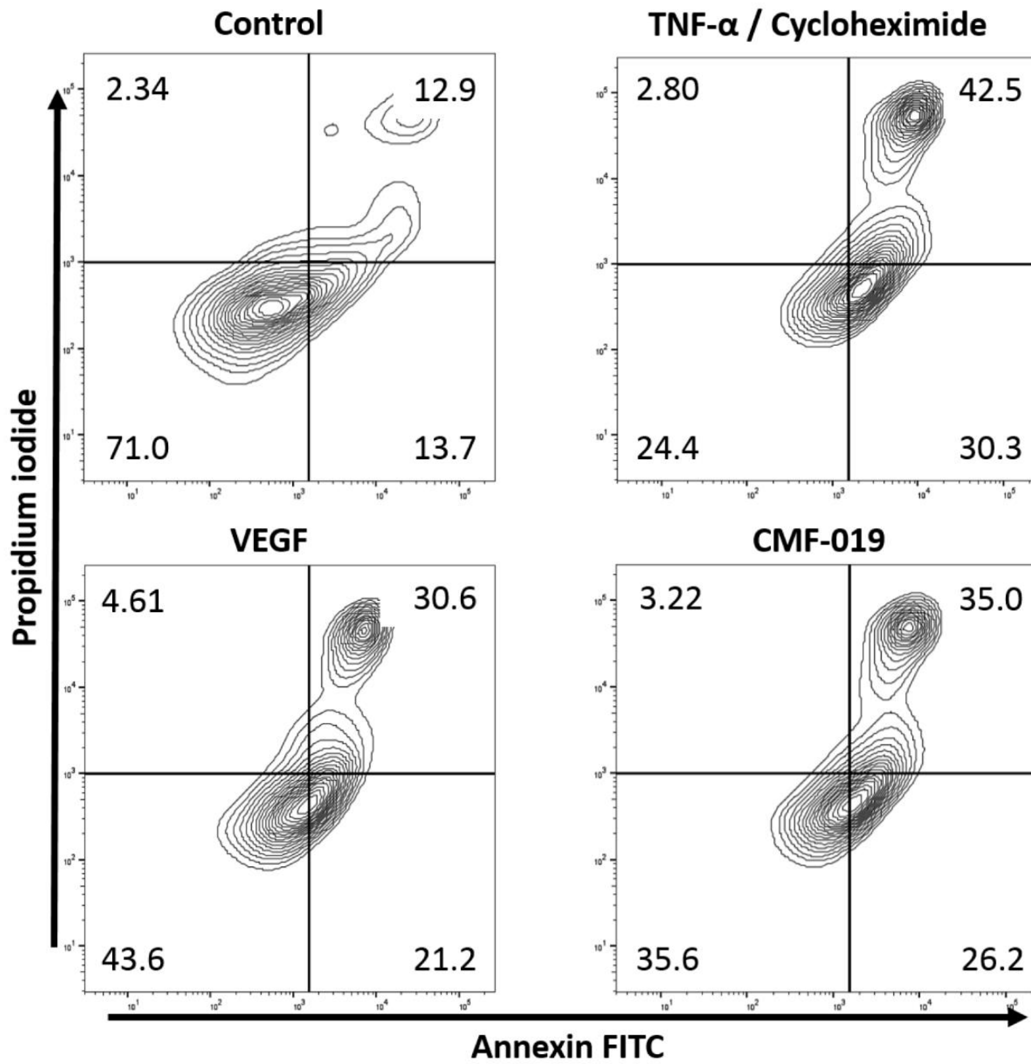
541 Figure 2



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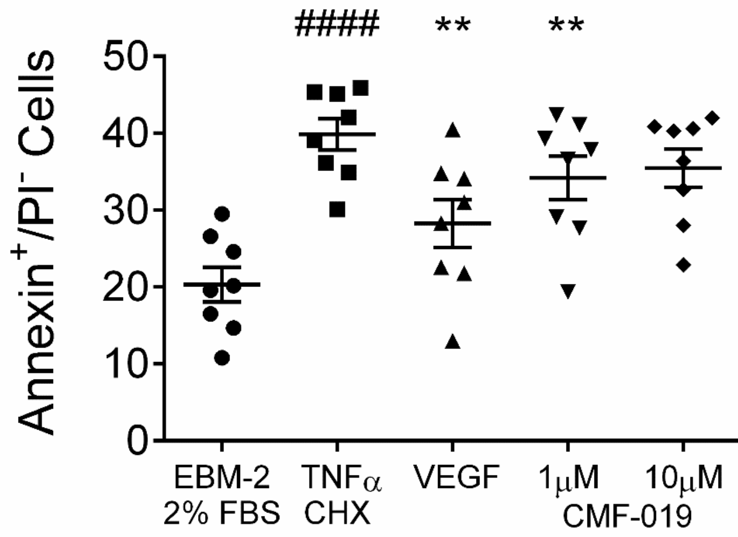
552 Figure 3



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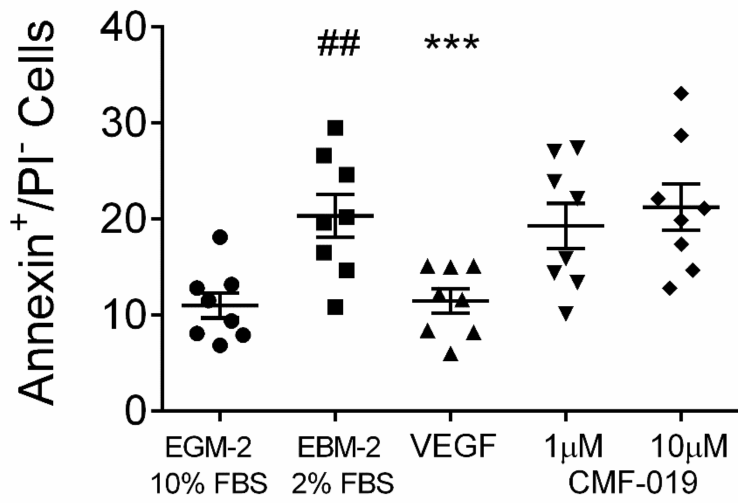
555 Figure 4



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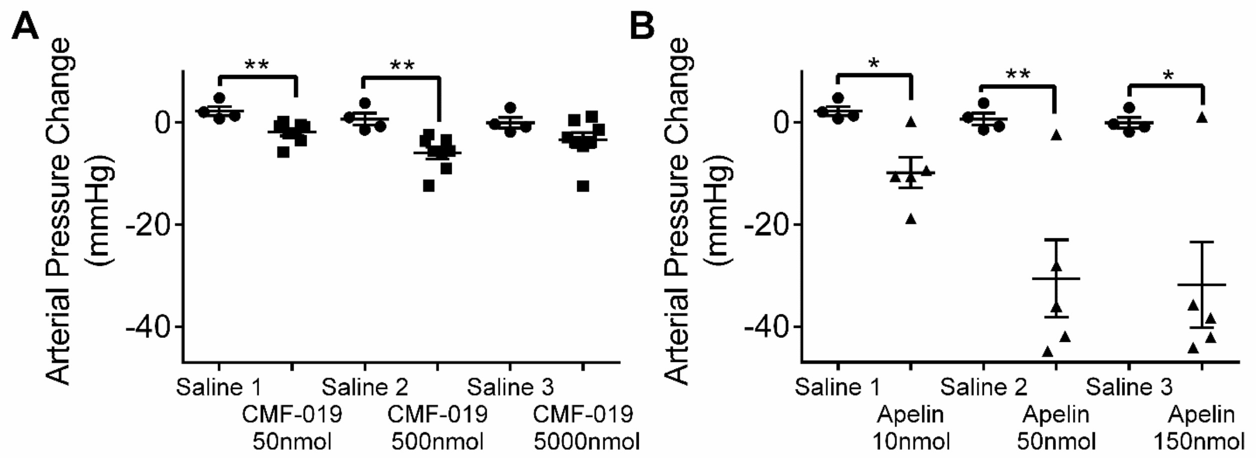
562 Figure 5



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567 Figure 6

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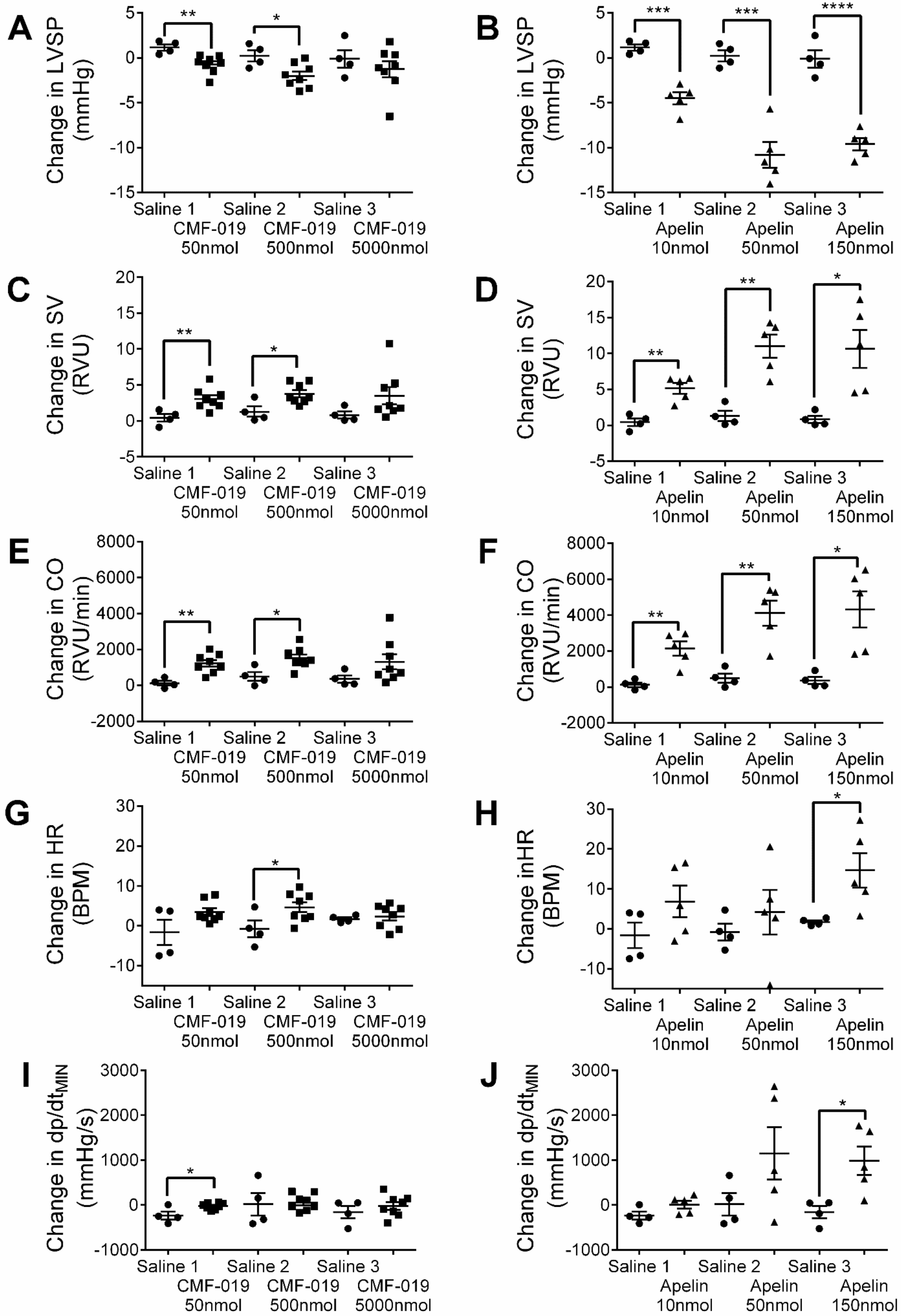

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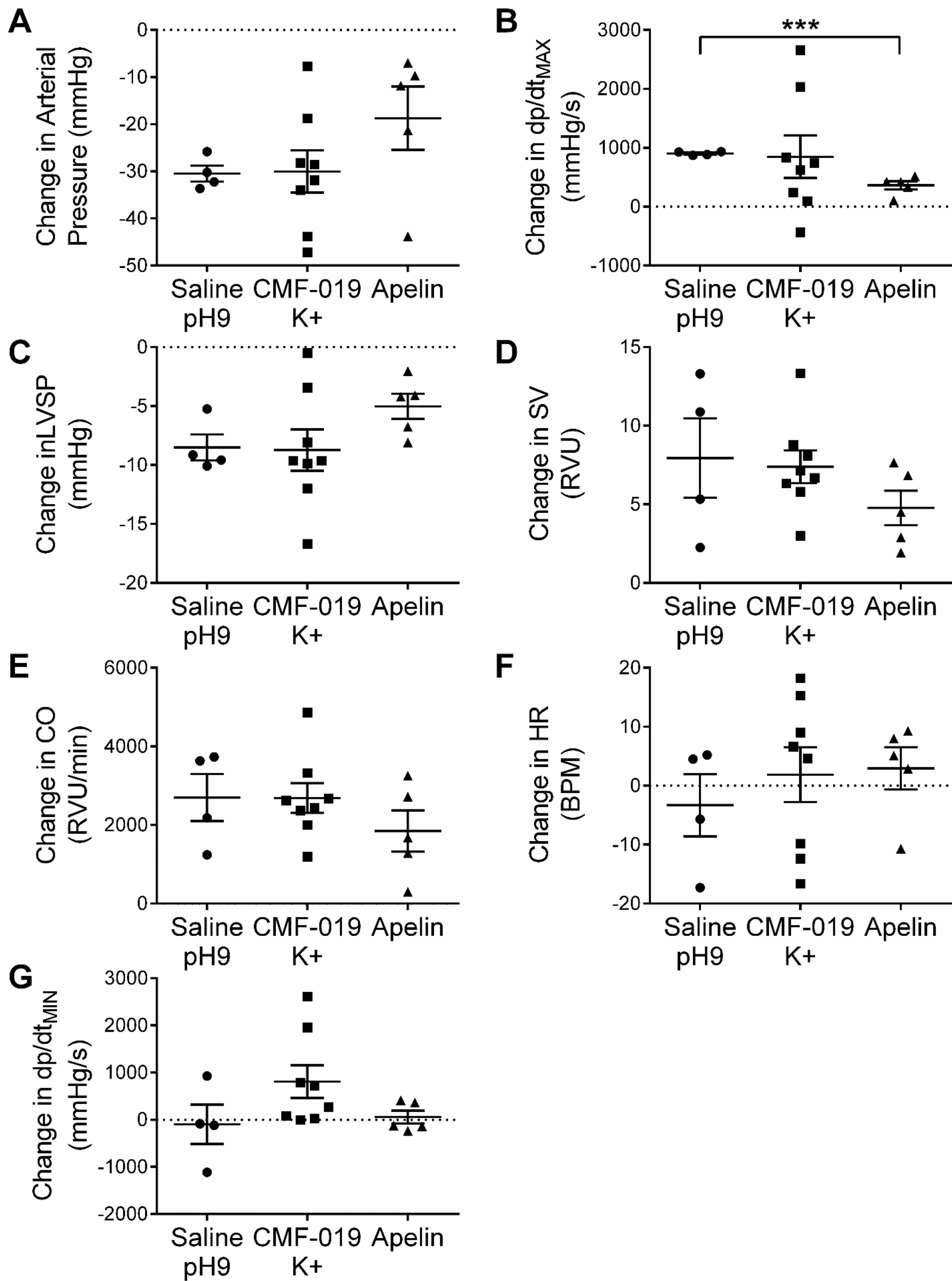
569 Figure 7



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