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Cardiovascular Effects of Urocortin-2: Pathophysiological Mechanisms and Therapeutic Potential

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Cardiovascular effects of urocortin-2: pathophysiological mechanisms and therapeutic potential

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A todos aqueles que me acompanharam nesta longa caminhada, contribuindo para o meu crescimento científico, profissional e pessoal.

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Cardiovascular Effects of Urocortin-2: Pathophysiological Mechanisms and Therapeutic Potential

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Abstract

Urocortin-2 (Ucn-2) is a peptide of the corticotrophin releasing factor-related family with several effects within the cardiovascular system. A variety of molecular mechanisms has been proposed to underlie some of these effects, although others remain mostly hypothetical. Growing interest in the cardiovascular properties of this peptide promoted several pre-clinical studies in the settings of heart failure and ischemia, as well as some experiments in the fields of systemic and pulmonary arterial hypertension. Most of these studies report promising results, with Ucn-2 showing therapeutic potential in these settings, and few clinical trials to date are trying to translate this potential to human cardiovascular disease. Ucn-2 also appears to have potential as a biomarker of diagnostic/prognostic relevance in cardiovascular disease, this being a recent field in the study of this peptide needing further corroboration. Regarding the increasing amount of evidence in Ucn-2 investigation, this work aims to make an updated review on its cardiovascular effects, molecular mechanisms of action and therapeutic potential, as well as to identify some research barriers and gaps in the study of this cardioprotective peptide.

Keywords: urocortin-2, cardiac function, heart failure, cardioprotection

CARDIOVASCULAR EFFECTS OF UROCORTIN-2: PATHOPHYSIOLOGICAL MECHANISMS AND THERAPEUTIC POTENTIAL

Urocortin-2 (Ucn-2) is one of the latest discovered peptides of the corticotrophin releasing factor (CRF)-related family. Until now, this family is composed of four members in vertebrates, coded by four distinct genes which appear to be highly conserved during evolution [1]. In 1955, CRF was discovered as the first mediator of stress response [2, 3], but it was only until 1995 that the first of the 3 urocortins (Ucns) known to date, urocortin-1 (Ucn-1), was isolated [4]. Growing interest on the pharmacological properties of Ucns leaded to the discovery of stresscopin and stresscopin-related peptide (SRP), homologues of urocortin-3 (Ucn-3) and Ucn-2, respectively [5].

This work focuses on Ucn-2, a 38-amino-acid (aa) peptide discovered in 2001 that stood out for its promising effects within the cardiovascular system, reported in multiple animal and human studies [6-8]. It aims to make an updated review of its cardiovascular effects, therapeutic potential, mechanisms of action and limitations in the current knowledge needing further elucidation.

Distribution and Structure

In both humans and animals, Ucn-2 is widely expressed throughout the body. It has a similar distribution to that of Ucn-1, being found in the cardiorespiratory, gastrointestinal, hematologic, endocrine, reproductive, urinary and central nervous systems, as well as in skeletal muscle, brown fat and skin [9-15].

Human Ucn-2 (hUcn-2) is coded by a gene with 621 base pairs (bp) from chromosome 3 and shares 76% of homology with mouse Ucn-2 (mUcn-2) at the amino-acid level [16]. Ucn-2 gene transcription results in a precursor peptide that undergoes specific processing, giving rise to the bioactive peptide. Human SRP, a 43-aa peptide, was identified from the same gene as Ucn-2, but with different interpretation of the cleavage sites from the precursor peptide [11].

A particular characteristic of both SRP and hUcn-2 appears to be somewhat baffling, which is the lack of a proteolytic site that would allow for C-terminal processing. In fact, although α-amidation of the C-terminal is commonly a requisite for generation of a bioactive peptide, hUcn-2, with a rather unusual C-terminal, does not appear to have a consensus amidation sequence [16, 9, 17]. Therefore, while the processing of the murine precursor peptide occurs with cleavage after the signal peptide, glycosylation and processing at the C-terminal (giving origin to an amidated protein), the human's form only goes through the first two phases, with the C-terminus remaining unmodified [17]. The natural cleavage site for hUcn-2 appears to be after Leu14 [17, 18].

Functionally, both Ucn-2 N- and C- terminals seem to be responsible for its receptor selectivity [19]. The middle region, while not appearing to have an influence on selectivity for corticotrophin releasing factor receptor-2 (CRF-R2), ensures correct positioning of N- and C- terminals with the CRF-R2 and it also harvests the binding site for corticotrophin releasing factor-binding protein (CRF-BP) [20]. This region may arise as an important target since modifications in the hUcn-2 peptide that reduce its affinity to CRF-BP conceivably increase its pharmacological activity [20]. Ucn-2 exists as a highly glycosylated precursor, what may function as a way of increasing its bioavailability [17].

Receptors

Since its discovery, Ucn-2 was found to be a strong agonist of CRF-R2, for both α and β isoforms, with low or no affinity for corticotrophin releasing factor receptor-1 (CRF-R1) [9].

The CRF-R2 gene was assigned to human chromosome 7p21–p15 in 1997 [21]. In mouse, it was found to be located on the proximal end of chromosome 6, which is consistent with the high degree of homology of this region with human chromosome 7 [22]. The gene has 16 exons and depending on alternative splicing at the 5' end gives rise to the three known variants of CRF-R2 identified in humans: CRHR2-α, CRHR2-β and CRHR2-γ (mice do not possess the γ variant) [5].

Contrasting to what happens in rodents, the major CRF-R2 isoform in human heart and skeletal muscle is CRF-R2α, whereas CRF-R2β plays a minor role [23]. More specifically, CRF-R2α mRNA is highly expressed in all four human cardiac chambers, whereas CRF-R2β mRNA expression predominates in left atrium showing general weak expression [24].

Both CRF-R1 and CRF-R2 belong to the B1 family of G-protein coupled receptors, possessing a N-terminal extracellular domain (ECD) linked to a 7-transmembrane helical bundle domain [25]. CRF-R2 corresponds to a splicing variant of CRF-R1, having an extra 29 aa inserted in the first intracellular loop (ICL) [26].

Peptide binding and receptor activation occur in two steps: primarily, the C-terminal portion of the peptide binds to the ECD, where disulfide bonds are critical for ligand recognition, and subsequently the N-terminal portion binds to the transmembrane domain initiating the intracellular signaling cascade [25]. This additional interaction happens specifically in the juxta-membrane domain of the receptor, which also contributes for its selectivity pattern, stabilizing affinity for Ucn-2 by about 30-fold in contrast with CRF-R1. The third ICL is believed to have a pivotal role in G-protein coupling and signal transduction [27, 28].

Cardiovascular Ucn-2 effects in physiological states

1) Cardiac effects

   a) Left Ventricular Function

      i) Animal Studies

Bale, Hoshijima et al. [29] observed acute intravenous (iv) injection of Ucn-2 in mice resulted in potent positive inotropic effects, as shown by the rise in peak (+) dP/dt and increased slope of the left ventricular end-systolic pressure-volume relation (ESPVR) for any given heart rate (HR). Ucn-2 injection also produced a decline in maximum left ventricular (LV) pressure and accelerated isovolumic relaxation, therefore enhancing diastolic function. These improvements of inotropism and lusitropism leaded to a clear enhancement in ventricular function, as demonstrated by the rises in stroke volume (SV), LV ejection fraction (EF) and the average 27% increase in cardiac output (CO).

In wild-type (WT) mice isolated cardiomyocytes, Ucn-2 increased maximal velocity of shortening, relengthening, peak height and shortening, as well as amplitude and fall in mean constant of

Ca^{2+} transient decay (Tau), reinforcing its positive inotropic and lusitropic effects [30]. Similar dose-dependent inotropic and lusitropic effects were further obtained in mouse ventricular myocytes [31], isolated rabbit myocytes [32], feline isolated left ventricular myocytes [33] and anaesthetized pig [34]. In the sheep normal heart, Ucn-2 iv injection caused a marked increase in CO, accompanied by a minor decrease in left atrial pressure (LAP) [35].

As shown by *in vivo* and *ex vivo* experiments performed by Gao, Lai et al.[36], Ucn-2 gene transfer in healthy mice may lead to potent, long lasting, positive inotropic and lusitropic effects, with improvements in ventricular function up to 7 months of gene transfer. In a comparative study of Ucn-2 *versus* Ucn-3 gene transfer effects, Giamouridis, Gao et al. [37] reported increases in ESPVR slope by 90% (vs 63% with Ucn-3), CO by 65% (vs 50% with Ucn3) and a 31% drop in Tau (vs 24% with Ucn3) after obtaining chronically elevated levels of Ucn-2. Tsuda, Takefuji et al. [38] implanted healthy mice with osmotic pumps, supplying a sustained Ucn-2 infusion for 4 weeks. The results were conflicting with the previous reports, with the emergence of cardiac dysfunction. The use of osmotic pumps does not allow for exogenous Ucn-2 temperature control, which may affect the peptide stability. On the other hand, continuously elevated Ucn-2 plasma levels opposed to Ucn-2 given in pulses may produce deregulated activation of several signaling pathways, which may associate with the deleterious effects found in this study.

ii) Human Studies

There are few studies on Ucn-2 cardiovascular actions in healthy humans. Two of them were performed *in vivo*, through an iv infusion of Ucn-2. The results were overlapping, with dose-related improvements in echocardiographic markers of ventricular function and contractility [39, 40]. One must take into account these works lack loading and HR control, limiting direct conclusions of inotropism. To clarify whether the improvements in left ventricular function are the result of Ucn-2 direct positive inotropic properties in humans, *in vitro* studies are needed. Yang, Kockskämper et al. [31] adressed this necessity, directly objectifying Ucn-2 positive inotropic effects in humans, denoted through a 15% increase in twitch force in isolated atrial trabeculae.

iii) Signaling Pathways

Yang, Kockskämper et al. [32, 31] performed studies in isolated rabbit and mouse myocytes suggesting the beforehand mentioned effects induced by Ucn-2 are mediated by actions in Ca^{2+} dynamics. In these works, Ucn-2 was shown to accelerate and increase the amplitude of Ca^{2+} transients (increasing not only systolic $_i[Ca^{2+}]$, but also diastolic $_i[Ca^{2+}]$), also with a marked acceleration of the decaying phase. The authors point CRF-R2-activated Gs-cAMP-PKA signaling cascade as one of the main pathways mediating these effects. PKA activation ultimately leads to phosphorylation of known Ca^{2+} regulatory proteins, such as L-type Ca^{2+} channels, ryanodine receptor channels and phospholamban. These mechanisms increase L-type Ca^{2+} current and SERCA activity, with concomitant rise in sarcoplasmic reticulum (SR) Ca^{2+} load and fractional SR Ca^{2+} release. The latest study has also evidenced the relevance

of the CaMKII pathway, which may be stimulated by an unclear PKA-independent mechanism, as well as by the rise in $_{i}[Ca^{2+}]$ induced by PKA. The conclusion that arises is that CaMKII and PKA signaling cascades work synergistically, regulating the phosphorylation of Ca^{2+} regulatory proteins, both being of central importance in the inotropic and lusitropic effects produced by Ucn-2.

A posterior study by Chen, Wang et al. [30] in mouse isolated cardiac myocytes, while further acknowledging the important role played by cAMP-PKA pathway, stands out AMPK pathway as another relevant mediator of Ucn-2 effects on Ca^{2+} dynamics and inotropism. As the authors note, AMPK pathway is stimulated by Ucn-2 treatment and has the potential to increase Ca^{2+} sensitivity and contractility of cardiomyocytes through cardiac troponin I phosphorylation. The mechanisms of interaction and regulation between AMPK and PKA-signaling pathways are, however, still unclear.

Walther, Pluteanu et al. [41] demonstrated that there is an increase in nitric oxide (NO) production induced by Ucn-2 in rabbit isolated cardiomyocytes, and this effect is linked to its inotropic effects. In their work, at least two pathways were shown to be associated with endothelial NO synthase (eNOS) activation: cAMP-PKA and PI3K-PDK-Akt signaling cascades. As shown by the authors, these pathways do not appear to interact directly, but they converge in phosphorylation of eNOS at Ser1177, ultimately leading to increased NO production. As expected, this culminates in stimulation of soluble guanil cyclase, increasing intracellular levels of cGMP. In the same study, the authors found Ucn-2 also stimulated MEK1/2-ERK1/2 pathway, but it did not play a role in the eNOS-mediated NO signaling in cardiac myocytes.

b) Chronotropism

In vivo iv injection of Ucn-2 exerts a dose-dependent positive chronotropic effect in mice, rat, sheep and humans [29, 42, 35, 39, 40]. Gao, Lai et al. [36], using AAV8.CBA.Ucn-2, performed *in vivo* studies reproducing the increase in HR seen in the previously mentioned works up to 7 months after Ucn-2 gene transfer. However, when Ucn2 administration was performed in isolated perfused hearts, the studies revealed no group differences, indicating that chronotropic effects seen *in vivo* are probably of reflex origin other than an intrinsic effect of Ucn2 gene transfer [36]. Studies in conscious rats corroborate this conclusion, since sympathetic and parasympathetic blockage by propranolol plus atropine abolished the tachycardic effect of Ucn-2 administration [43, 44]. In sheep, Ucn-2 seems to have a direct chronotropic effect, independent of autonomic and baroreceptor activation [45]. In fact, in this species, Ucn-2 produces a sustained decrease in the sympathetic traffic to the heart while there is still increased HR, weakening the hypothesis of this effect arising only from baroreflex activation [45].

c) Cardiac Morphology and Electrical Activity

As reviewed bellow, when in the presence of a pathologic condition which may ultimately lead to hypertrophy, Ucn-2 appears to have an obvious anti-hypertrophic effect. However, the effects in the normal heart seem to be less clear, with little conformity when analyzing the effects *in vitro* and *in vivo*. Indeed, *in vitro* works in rat neonatal isolated cardiomyocytes assuredly indicate towards a pro-hypertrophic effect of Ucn-2 in the healthy heart. As a matter of fact, incubation with SRP/Ucn-2 was shown to increase

cardiomyocyte size, protein to DNA ratio and the incorporation of [3H]-leucine, all markers of cardiac hypertrophy [46, 47]. On the other hand, *in vivo* studies accessing the effects of chronically elevated levels of Ucn-2 obtained through gene transfer seem to favor an anti-hypertrophic effect [36, 37]. Necropsy analysis indicated less hypertrophy in AAV8.CBA.Ucn2 transfected mice, with reduction in body weight-adjusted LV weight [36, 37]. Contrasting with these works, Tsuda, Takefuji et al.[38] evidenced a pro-hypertrophic effect after chronic Ucn-2 ministration in control mice, which is in line with the above mentioned emergence of cardiac disfunction found in the same study.

Yang, Kockskämper et al. [31], using both mouse ventricular myocytes and myocardium from right-atrium of patients undergoing cardiac surgery , showed an association of Ucn-2 with arrhythmogenic events. Noteworthy, spontaneous Ca^{2+} increases where observed and preceded the shortenings, pointing out release from SR as the inherent mechanism. *In vivo,* only one study briefly mentions Ucn-2 effects in electrocardiographic parameters in healthy humans, finding no relation with arrhythmogenic events [39]. In other works testing Ucn-2 in healthy animals and humans there is no reference to electrocardiographic parameters. Nonetheless, there is no report of symptomatic arrhythmias and/or relevant electric events in the studies in which electrocardiographic monitoring was performed.

i) Signaling Pathways

Walther, Awad et al. [48] attempted to explain the mechanisms behind the pro-hypertrophic effect of Ucn-2 observed *in vitro*. The authors found Ucn-2 can disturb the equilibrium between transcription factors of maintenance (NFAT1c) and "maladptative" (NFAT3) hypertrophy. Indeed, they observed Ucn-2 induced nuclear export of NFATc1 to cytosol, while not altering the location of NFAT3. They also suggested the mechanism involved might be the Ucn-2-stimulated PI3K/Akt/eNOS/NO pathway. Activation of this pathway would ultimately lead to PKG1 activation by cGMP, also involving activation of other kinases (GSK3β, JNK, p38), resulting in phosphorylation, deactivation, and nuclear export of NFAT. A previous study in rat neonatal isolated cardiomyocytes also suggested the hypertrophic effect induced by Ucn-2 in the normal heart was promoted by PI3K/Akt pathway [46].

Chronic activation of Ucn-2 induced pathways, such as PKA, CAMKII and PI3K/Akt, may explain the pro-hypertrophic effects observed *in vivo* in control mice [38]. On the other hand, the apparent anti-hypertrophic effect observed after Ucn-2 gene transfer *in vivo* was suggested to be a result of renin–angiotensin–aldosterone system (RAAS) inhibition by Ucn-2 [36].

In the first study accessing the direct electrophysiological effect of Ucn-2 in action potential, Yang and Zhu [49] found Ucn-2 significantly prolonged action potential duration in a time and concentration-dependent manner in guinea pig isolated myocytes. The elongation of action potential induced by Ucn-2 may lead to changes in refractory period and this may be one of the explanations for the beforehand described arrhythmogenic effect of high Ucn-2 doses *in vitro*. The authors found this effect appeared to be mediated by PKA activation and Ca^{2+}-dependent activation of Na^{+}/Ca^{2+} exchange (NCX), as well as increased IKr inhibition rate and reduced IKr current. The inhibition of IKr was not mediated by PKA signaling and the contribution of other Ucn-2 activated pathways was not evaluated yet. The authors also

noticed Ucn-2 induced a right shift in the threshold for IKs current activation and decelerated rate of IKs activation.

2) Vascular Effects

a) Animal Studies

In vitro, increasing doses of Ucn-2 were shown to express a dose and time-dependent vasodilatory effect, similar in potency to that of Ucn-3, both being more potent than CRF, but less potent than Ucn-1 [50].

In vivo, the effects tend to vary according to the species and vascular bed. In rats, several studies have shown consistent dose-dependent decreases in blood pressure (BP) after Ucn-2 infusion [51, 42-44, 52]. In this specie, Ucn-2 appears to induce marked mesenteric vasodilatation, while it produces an inconsistent renal vasodilation, indicating less powerful coupling of CRF-R2 receptors in the renal vascular bed. In mice, Ucn-2 was found to be a potent vasodilator, with decreases in arterial elastance and resistance, as well as in systolic, diastolic and mean BP [29, 37]. In pig, intra-coronary infusion of Ucn-2 leaded to an approximate 15% increase in coronary blood flow [34]. Contrastingly, in healthy sheep, Ucn-2 iv injection resulted in a minor reduction in calculated total peripheral resistance (CTPR). On top of that, although an initial drop in mean arterial pressure (MAP) was found, the overall effect was elevation of MAP [35].

b) Human Studies

Wiley and Davenport [27] conducted *in vitro* studies with human internal mammary arteries showing a potent vasodilatory effect of Ucn-2, with approximately 61% reversal of endothelin-1 (ET-1)-induced vasoconstriction. More recently, in coronary arteries dissected from patients subjected to heart transplantation, Ucn-2 induced marked relaxation in both intact endothelium and endothelium-denuded arteries, with no significant differences between the two [53].

Venkatasubramanian, Griffiths et al. [54] performed intra-brachial infusions of Ucn-2 in healthy volunteers, resulting in a pronounced dose-dependent vasodilation in the infused arm's arteries. No changes were noticed in blood flow in the non-infused arm or in systolic blood pressure (SBP). The authors also describe no signs of tachyphylaxis associated with locally induced vasodilatation. Similar effects were obtained in a posterior study after intra-brachial infusion of Ucn-2 in both healthy subjects and heart failure (HF) patients [55]. In the latter study, systemic iv delivery of Ucn-2 in healthy subjects leaded to significant falls in MAP and peripheral vascular resistance index, accompanied by rises in HR.

c) Endothelium-dependency and Signaling Pathways

The endothelium-dependency of Ucn-2 vasodilatory effect presumably varies according to the species and the vascular bed. In humans, *in vitro* studies in human internal mammary and coronary arteries indicate towards a potent endothelium-independent vasodilatory effect of Ucn-2, although there is also

evidence of eNOS activation contribution *in vivo* [27, 53, 54]. As to other species, Gardiner, March et al. [43] found no involvement of NO or prostanoids in Ucn-2-induced vasodilation in conscious rats. In pig, on the other hand, the increase in coronary blood flow induced by Ucn-2 appears to be NO-dependent, suggesting the dependence on endothelium [34].

As to the signaling pathways involved in vasodilation, cAMP-PKA signaling was shown to have an important role in Ucn-2 induced vasodilation, also with contribution of p38/MAPK pathway (speculated to lead to production of vasodilatory prostaglandins) [50]. In porcine isolated aortic endothelial cells, NO generation stimulated by Ucn-2 appears to be mediated by cAMP-PKA and CaMKII signaling pathways, as well as increased cytoplasmic [Ca^{2+}] [56, 57]. As shown by Grossini et al. [57], Ucn-2 seems to raise levels of cytoplasmic [Ca^{2+}] through two mechanisms: influx from intracellular pools and extracellular medium and membrane hyperpolarization as a consequence of K^+ channels opening.

Therapeutic potential in cardiovascular disease

1. Heart Failure

a) Animal Studies

The first studies assessing Ucn-2 effects in failing hearts focused on documenting its acute effects in animal models of HF. In line with this, Bale, Hoshijima et al. [29] reported improvements in left ventricular diastolic and systolic functions in MLP-deficient cardiomyopathic mice after an iv bolus of Ucn-2. Rademaker, Cameron et al. [35] uncovered similar outcomes when using incremental iv boluses of mUcn-2 in an ovine model of HF. Moreover, they found a marked fall in CTPR and an overall reduction in MAP, contrasting with the findings from healthy sheep. A plausible explanation would be that in the HF model the arterial vasculature is in a pre-constricted state, which would enhance the vasodilatory properties of Ucn-2. The authors also reported important hormonal effects following Ucn-2 injection (detailed in Table I).

Rising interest in the therapeutic potential of Ucn-2 in this setting led a number of investigation groups to start exploring the effect of Ucn-2 given therapeutically. In a sheep model of chronic HF, a 4-day iv infusion of mUcn-2 presented with numerous favorable effects, improving cardiac function, hemodynamics, hormonal response and renal function [58]. On the other hand, potentially adverse effects of increased cortisol levels from secondary HPA axis stimulation and decreased food intake were only transient. In the same model of ovine HF using mUcn-2 administered through a pulmonary artery catheter, similar acute effects were observed in LV function, peripheral resistance, neurohormonal response and renal function [59]. Ucn-2 was also tested in the setting of acutely decompensated heart failure (ADHF) in ovines, resulting in a clear boost in cardiac function, suppression of potentially harmful neuro-hormonal response and amelioration of renal function [60]. The authors also documented lessened genetic expression of RAAS, as well as fibrosis, hypertrophy, apoptosis and inflammation markers. Histologically, Ucn-2 decreased collagen deposition in the heart and kidney.

Given that Ucn-2 has a rather short half-life, Lai, Gao et al. [61] made an effort to overcome this issue by performing an Ucn-2 gene transfer after induction of HF in mice. The gene transfer was attained by a single iv injection of a viral vector (AAV8) encoding Ucn-2 gene (AAV8.UCn2), giving rise to a 70-fold increase in LV Ucn-2 mRNA 5 weeks after injection. At this time, the authors reported an improvement in LV systolic and diastolic functions, with no group differences in MAP. The results also pointed to an anti-hypertrophic effect, with a reduced left ventricle/body weight ratio in treated animals. These findings also extended to Ucn-3 [62]. Ucn-2 gene transfer in Western-Diet (WD)-fed mice was also able to significantly improve glucose tolerance, with reduced fasting blood glucose and increased glucose disposal [63]. At the same time, it improved both diastolic and systolic LV functions and decreased liver fatty infiltration in mice with WD-induced diabetes-related LV dysfunction [63]. This highlights Ucn-2 as a potential therapeutic weapon in the setting diabetes-induced heart disease, targeting both glucose intolerance and cardiac dysfunction.

i. Ucn-2 in combination with established HF therapies

In order to prospect the realistic therapeutic potential of Ucn-2, Rademaker, Charles et al. performed a number of studies using Ucn-2 in combination with other drugs commonly used in the setting of HF, namely angiotensin converting enzyme inhibitor (ACEI) [64], loop diuretic [65], β-blocker [59] and mineralocorticoid receptor antagonist (MRA) [66]. All the works were performed in sheep with pacing-induced HF.

In the first study, the authors studied the effects of intra-arterial infusions of Ucn2 both isolated and in combination with the ACEI captopril [64]. They came to the conclusion that the addition of Ucn-2 to an ACEI may have beneficial effects on hemodynamic, hormonal and renal responses, to a greater extent than with ACEI treatment alone. Inclusively, in the matter of renal function, Ucn-2 co-treatment with ACEI appears to have the potential to neutralize some of the undesired effects observed with ACEI treatment alone, namely the reduction in creatinine clearance and urine output. Ucn-2 addition to ACEI was also able to neutralize the potentially adverse rise in plasma renin activity (PRA) induced by the latter.

Using the same regimen as in the previous work, Rademaker, Charles et al. [65] described Ucn-2 effects in combination with furosemide. The findings were compatible with an overall improvement of furosemide infusion effects. Specifically, the co-infusion resulted in enhanced renal function without additional K^+ excretion, suppression of PRA and greater and more sustained decrease in aldosterone than seen with both treatments alone. Given separately, Ucn-2 improved CO and hemodynamics, in accordance with previous studies in the same animal model of HF.

Employing a similar study protocol, the authors then accessed the effects of Ucn-2 in isolation and in addition to the β-blocker metoprolol [59]. The results were once again promising, with Ucn-2 avoiding the bradycardia, fall in CO and increase in CTPR induced by β-blocker therapy [59]. Ucn-2 may, therefore, have the appealing effect of increasing β-blocker tolerability, possibly allowing for an earlier and safest introduction in the setting of ADHF.

Lastly, the authors tested the effect of intra-arterial infusions of Ucn2, both isolated and in combination with a mineralocorticoid receptor antagonist (MRA) [66]. With combined therapy, the effects

of increased LV function, decreased LAP and CTPR were observed at a similar degree to those incited by Ucn-2 alone [66]. When added to a MRA, Ucn-2 also had the ability of improving the neuro-hormonal response and renal function, reducing PRA, angiotensin II and K^+ levels [66]. Separate administration of MRA increased plasma aldosterone, and Ucn-2 was capable of smoothing this response in co-therapy [66]. Overall, this study unveils possible beneficial effects of adding Ucn-2 to MRA therapy, with the particularly enticing effect of virtually inhibiting hyperkaliemia, a well-known dangerous side effect of MRA.

Detailed information on the beforehand mentioned pre-clinical studies can be found on Table I.

b) Human studies

Davis, Pemberton et al. [67] studied the actions of iv hUcn-2 given to eight males suffering from congestive HF with reduced ejection fraction. They documented dose-dependent improvements in cardiac function and falls in SBP, DBP and MAP. They also noticed a slight reduction in urine volume and natriuresis, with no neurohormonal responses worth of consideration. This contradicts findings from ovine HF and is probably related to the deeper severity of disease and neurohormonal deregulation in this animal model. It seems likely that the natriuretic effect of Ucn-2 is only significant when there is an important background of RAAS activation and Na^+ retention. Regarding side effects, Ucn-2 leaded to flushing in all subjects, persisting within 2 hours after the infusion.

Chan, Frampton et al. [68] performed a double-blind, placebo-controlled clinical trial in patients admitted for ADHF, in which they studied the effect of Ucn-2 iv infusion as an adjunct to the conventional therapy initiated by the physician. Ucn-2 induced a prompt and marked hypotensive effect, as well as an increase in CO, although the latter was not sustained. The infusion transiently worsened renal function and increased PRA. Again, these findings diverge from those obtained in ovine HF. The emergence of reflex responses to the marked fall in BP and consequent decrease in renal perfusion may partially explain these discrepancies. The adverse effect of flushing was observed with Ucn-2 as previously documented, although it was not noticed by the patients. The peak plasma levels achieved in this study were twice the concentration recorded in the previous work in stable HF patients, remaining the question of the greater clinical applicability of this peptide in this scenario at a lower dose.

Stirrat, Venkatasubramanian et al. [55] conducted a double-blind, placebo-controlled cross-over study with iv infusions of either placebo or SNP, followed by either Ucn-2 or Ucn-3. They noticed increases in cardiac index, with concomitant falls in MAP and peripheral resistance index. Hypotension reaching sopping criteria occurred in one patient out of nine, once more rising the concern for important hypotension after Ucn-2 infusion in HF patients.

Table II harvests detailed information on the clinical trials using Ucn-2.

c) Signaling Pathways

Several mechanisms have been suggested to explain the positive inotropic and lusitropic effects of Ucn-2 in healthy hearts. These effects are also presumably activated after Ucn-2 administration in the setting of HF and justify, at least partly, the clear improvement in cardiac function observed in the several studies reviewed above. These mechanisms are explained in detail in the section "Left Ventricular Function" (subsection "Signaling pathways").

Specifically in the failing heart, Lai, Gao et al. [61] documented effects in Ca^{2+} dynamics, reduced phosphorylation of CAMKII, and increased cardiac myosin light chain kinase expression as potential mechanisms leading to the improvement in heart function induced by Ucn-2. In the matter of Ca^{2+} dynamics, the findings were in line with those previously described in non-failing isolated myocytes [32, 31]. Otherwise, reduced phosphorylation of CAMKII contrasts with findings from normal hearts, where activation of the CAMKII pathway appears to be one of the mechanisms mediating Ucn-2 cardiac effects [31].

As we can conclude from the pre-clinical studies exposed above, Ucn-2 appears to exhibit anti-hypertrophic properties in failing hearts. Walther, Awad et al. [48] proposed shifting of NFAT transcription factor isoforms balance towards the physiological state as one of the potential mechanisms mediating this effect. In their work in myocytes from rabbits with non-ischemic HF, Ucn-2 induced nuclear export of NFAT3, an isoform overexpressed in the nucleus in HF and associated with cardiac hypertrophy [48]. *In vivo*, there is less knowledge regarding the mechanisms involved in the anti-hypertrophic effect of Ucn-2. On one hand, the beneficial hormonal effects extensively evidenced from works in sheep HF may intuitively play a part. On the other hand, the significant improvement in hemodynamics alone could account for the reduction in cardiac hypertrophy and fibrosis in HF. Even so, despite having similar outcomes on hemodynamics when compared to Ucn-2, dobutamine was not able to mimic Ucn-2 effects of reducing cardiac fibrosis and hypertrophy markers, suggesting it possesses direct anti-hypertrophic properties [60].

Although this hypothesis was not tested in HF, the sustained reduction of sympathetic drive to the heart observed in healthy sheep may antagonize the harmful and potentially pro-arrhythmic sympathetic overdrive of cardiac dysfunction, arising as a potential additional mechanism explaining Ucn-2 beneficial effects in cardiac disease.

2. Ischemic Disease

The first study addressing Ucn-2 as a potential cardioprotective peptide was performed in neonatal rat ventricular myocytes submitted to hypoxia and reoxygenation [69]. The authors used SRP (1nM) administered prior to subjecting the cells to hypoxia or at the onset of reoxygenation, reporting an enhanced viability of the cardiomyocytes. Brar, Jonassen et al. [70] recorded similar findings in mouse cardiomyocytes. *Ex vivo,* Ucn-2 ministration from the onset of reperfusion significantly reduced infarct size in rat hearts subjected to ischemia/reperfusion (I/R) [70]. In this setting, Ucn2 treatment given before

ischemia was also shown to distinctly improve cardiac function, with slightly less marked results when Ucn-2 was given only during reperfusion [71]. Similar findings were obtained in mice isolated hearts [72].

In vivo, several studies in animal models of ischemia have evidenced Ucn-2 therapeutic properties in this scenario. Liu, Yang et al. [73] observed a notable reduction in arrhythmias, allied to a 50% reduction in infarct size after iv Ucn-2 in a rat model of I/R. In male wistar rats submitted to left coronary artery ligation, an Ucn-2 iv injection before reperfusion was able to, not only limit infarct size and fibrosis, but also induce a sustained recovery of cardiac function in treated animals [74]. Likewise, an intraperitoneal Ucn-2 injection before induction of I/R injury in mice significantly restricted necrosis and improved contractile function in the previously ischemic area [72]. These results also applied to chronic Ucn-2 treatment, with the addition of left ventricle geometry improvement and significant attenuation of collagen 1 and β-myosin heavy chain gene expressions [75].

a) Signaling Pathways

Several mechanisms have been proposed to explain Ucn-2-mediated cardioprotection. One of these mechanisms is the phosphorylation of ERK1/2-P42,44, reported in works performed in both mouse [70] and rat [69] cardiomyocytes. The activation of this pathway seems to be mediated by MEK1 and Ras/Raf-1 kinase-dependent mechanisms, without intervention of PKA or cAMP [70]. Another suggested pathway is the attenuation of free-radical induced injury induced by Ucn-2 stimulation of anti-oxidative enzymes, such as superoxide dismutase and glutathione peroxidase [73]. Furthermore, Li, Qi et al. [72] highlighted the importance of Ucn2-CRFR2-PKCε -AMPK pathway to the pharmacological role of Ucn-2 in the setting of ischemia. They revealed the heart may secrete Ucn-2 in response to ischemia as an attempt to minimize cardiac damage, and AMPK also appears to be involved in this mechanism. Ucn-2 has also been shown to interfere with apoptotic pathways, inhibiting p38 and regulating Bcl-2 signaling towards an anti-apoptotic effect [71]. Specifically, Ucn-2 appears to induce Bcl-2 expression in the mitochondria, enhance Bim phosphorylation and decrease Bax phosphorylation, all of these actions translating into a pro-survival effect [71]. Ucn-2 has also been reported to prevent dysregulation in Ca^{2+} dynamics induced by ischemia, and this may be an additional mechanism through which it exerts its cardioprotective actions [74].

3. Systemic Arterial Hypertension

Given its clear vasodilating properties and direct effects in the heart, the hypothesis of Ucn-2 as a plausible therapeutic option in systemic arterial hypertension became worth of test.

Dieterle, Meili-Butz et al. [76] were the first to explore this hypothesis with an experimental study in Dahl salt-sensitive rats, which they treated with intra-peritoneal (ip) hUcn-2 for five weeks. They found an immediate reduction of SBP, not accompanied by reflex tachycardia, as well as a sustained decrease in BP in treated animals. They also evidenced an apparent anti-hypertrophic effect of Ucn-2, together with acute positive inotropic effects. In addition to its BP-lowering properties, Ucn-2 produced clear enhancements in diastolic and systolic functions in spontaneously hypertensive rats, suggesting a potential

benefit in terms of LV function and geometry in arterial hypertension [77]. In the Langendorff-perfused heart at the stage of hypertension-induced severe HF, Ucn-2 perfusion (5nM during 20 minutes) induced positive inotropic and lusitropic effects, together with coronary blood flow improvement [78]. At the same time, it leaded to a significant reduction of monophasic action potential duration and LV conduction time, with an increase in ventricular fibrillation threshold.

a) Signaling Pathways

The mechanisms through which Ucn-2 exerts its vasodilating properties and their relative relevance seem to vary with the specie and the vascular bed involved, and they were reported in the section "Vascular Effects" (subsection "Endothelium dependency and Signaling Pathways"). Most of these works were performed in non-diseased vessels, and weather the same mechanisms apply under pathologic conditions remains a question of relevance.

Studies focusing on the signaling mechanisms mediating Ucn-2 effects in animal models of hypertension are rather scarce, and the existing ones address solely the pathways through which Ucn-2 may improve cardiac function in hypertension-induced HF. In this matter, Meili-Butz, Bühler et al. [78] documented the improvement in contractile function seen acutely after Ucn-2 as a consequence of increased SR Ca^{2+} load. By its turn, Liu, Liu et al. [77] suggested Ucn-2 may relief the Ca^{2+} overload characterizing SHR, probably through inhibition of current density through L-type Ca^{2+} channels, and this may be a pathway through which it can improve LV geometry and function in this condition.

4. Pulmonary Arterial Hypertension

Recently, we reported Ucn-2 appears to display an overall favorable effect in Pulmonary Arterial Hypertension (PAH), accomplished by direct actions in both the pulmonary vasculature and right ventricle [79]. Indeed, Ucn-2 treated rats with monocrotaline-induced PAH exhibited higher percent of survival, as well as increased exercise tolerance and improved body weight. In addition, they presented with attenuated RV and pulmonary small arteries remodeling, improved biventricular systolic and diastolic functions and reduced levels of cardiac damage and fibrosis markers. In animals submitted to pulmonary artery binding, Ucn-2 was capable of undermining RV hypertrophy and fibrosis, indicating it exerts direct effects in the RV.

Details on pre-clinical studies using Ucn-2 in the conditions mentioned on 2., 3. and 4. can be found on Table III.

Ucn-2 potential as a biomarker in cardiovascular disease

A recent field in the study of Ucn-2 properties is focusing on its potential as a biomarker of diagnostic and prognostic relevance in cardiovascular disease. Although circulating levels of Ucn-2 in

healthy humans seem to be very low [39], a rising number of studies have found interesting associations between augmented Ucn-2 levels and conditions in the spectrum of cardiovascular disease.

Topal, Yağmur et al. [80] performed an observational study addressing this matter, gathering data from 86 hemodynamically stable outpatients. Ucn-2 serum levels in these patients were quantified by using Human Urocortin II ELISA Kit, with a detection range of 6.25-400 pg/mL. They found elevated levels of serum Ucn-2 in patients with mild to moderate systolic dysfunction, but not in severe ventricular dysfunction, indicating towards an increased secretion of Ucn-2 in early HF. There were no significant differences in Ucn-2 serum levels between patients with or without diastolic dysfunction. However, 73% of the patients in this group had only grade 1 diastolic dysfunction, remaining a question if Ucn-2 levels would remain unchanged in increasingly severe degrees of disease. In line with these findings, Tsuda, Takefuji et al. [38] found a median of 7.5-fold increase in Ucn-2 plasma levels in patients with non-ischemic dilated cardiomyopathy *versus* controls, again suggesting a possible relation between Ucn-2 levels and chronic HF. The only work acessing Ucn-2 plasma concentrations in patients with PAH found no significant differences in comparison with controls, although its mRNA levels were elevated in the RV of patients with RV failure when compared with non-failure patients [79].

Liew, Yandle et al. [18] published the first and single study to date using a high-sensitivity ELISA assay to measure a N-terminal pro-form of Ucn-2 (NT-proUcn-2) in human plasma. The authors extensively validated the assay, reporting a detection limit of 1.52 pmol/L (approximately 0.14 pg/mL) with an assay range of 4.3 – 102 pmol/L (1.17-27.8 pg/mL). Of relevance, this study shows increased NT-proUcn-2 plasma levels in patients suffering from HF, although this increase is rather modest when compared to the rise of NT-proBNP. Moreover, unlike plasma NT-proBNP, NT-proUcn-2 levels did not significantly differ between NYHA classes, but they showed an inverse relationship with 2-year mortality. This inverse relation is unique and somewhat intriguing since it is an uncommon finding among biomarkers in HF. Notwithstanding the lack of further corroboration and careful investigation, these works unveil a possible application of Ucn-2 as a prognostic marker in HF, which may proof substantially valuable.

In the field of vascular disease, Emeto, Moxon et al. [81] reported an association between aortic abdominal aneurism and Ucn-2 plasma levels, with a 4-fold rise in the prevalence of this condition in patients with Ucn-2 in the highest quartile.

It is relevant to notice that there is very little Ucn-2 assay validation to allow valid comparison between these studies and yield solid conclusions to wheather or not it can be used as a biomarker of clinic relevance in cardiovascular disease. Altough there is clear potential in the application of Ucn-2 as a biomarker for diagnosis and/or follow up in this conditions, further works are needed to corroborate previous findings and refine the situations in which this application may be of worth.

Future Directions and Concluding Remarks

Ucn-2 appears as a peptide of great potential in a multitude of conditions in the spectum of cardiovascular disease.

In fact, *in vitro*, *ex vivo* and *in vivo* works in numerous animal species as well as in humans show that Ucn-2 elicits potent positive inotropic and lusitropic effects, as shown by the improvement in both

echocardiographic and direct hemodynamic parameters of systolic and diastolic functions. A number of pre-clinical studies support its beneficial effects in several cardiovascular diseases which represent a great burden of disease worldwide. Therapeutically, it appears to directly improve cardiac function and exhibit intrinsic cardioprotective properties, having also important effects on the vasculature. Allied to these properties, it was shown to trigger favorable hormonal responses, down-regulating the deleterious hormonal response characterizing HF. It was also shown to positively interact with established mortality-reducing HF therapies.

These represent some of the advances in Ucn-2 investigation, but there were also some drawbacks when it comes to transitioning to clinical trials. In fact, there are few studies to date testing Ucn-2 ministration in human disease, all of them in the setting of HF. These studies report less encouraging results than those found in animal investigation, with the problem of hypotension ultimately leading to reduced renal perfusion and the resulting consequences. A plausible explanation could be that patients find themselves in less advanced stages of disease than the animal models. Additionally, the doses of Ucn-2 used appear to be inadequate to achieve the most advantageous balance between its direct cardiotropic effects and limiting vasodilating properties. Careful, evidenced-based, patient selection, combined with refined posology of ministration could contribute to overcoming the exposed problems and make progress in human applicability of this appealing peptide.

Although there seems to be sufficient evidence to encourage Ucn-2 investigation in human disease, there are still relevant questions left unanswered. For one, while we lack studies in physiological states to adress some of these questions, works in pathological conditions to clarify others are rather scarse. For example, there is some uncertainty when referring to Ucn-2 properties in terms of cardiac hypertrophy and arrhythmogenic potential under physiological conditions. At the same time, even though there is quite some evidence on the signaling pathways underlying Ucn-2 cardiovascular effects in the physiological state, most of these mechanisms were not tested under pathological conditions, and we do not know whether the same pathways apply in these situations. On the other hand, although we acknowledge Ucn-2 levels are elevated in human HF, the mechanisms promoting Ucn-2 secretion in this condition, as well as the cellular origin of the peptide remain unknown.

Lastly, although it is an appealing therapeutic option in the field of cardiovascular disease, there are still several gaps in Ucn-2 investigation. Given its potential, further works are needed to fill in these gaps, in order to take a step forward in achieving clinical applicability of Ucn-2.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Tables

Table I – Pre-clinical studies with Ucn-2 in Heart Failure – Part 1

Species	Model	Posology and route of ministration	Outcomes	Reference
Sheep	Pacing-induced HF	Incremental IV boluses of mUcn-2 (10, 50, and 100 µg) at 2-hour intervals	Increased CO; Decreased CTPR, LAP and MAP; Decreased PRA, ALD, ET-1, ANP, BNP, epinephrine and AVP; Increased corticotropin, cortisol and Ucn-1; Increased urine volume, uNa^+, uK^+, uCr and CrCl.	35
		4-day IV infusion of mUcn-2 (25 µg bolus + 0.75 µg/kg/h)	Increased dP/dt(max) and CO; Decreased CTPR, LAP and MAP; Decreased PRA, ALD, ET-1, ANP, BNP and AVP; Decreased plasma Na^+ and Cr; Increased urine output (acutely), uNa^+, uCr and CrCl; Transiently decreased food intake.	58
		mUcn2 infusion through PAC (50 µg bolus+ 50 µg/h) both isolated and in combination with metoprolol (10mg slow bolus + 10mg/h)	**Isolated Ucn-2:** Increased HR, CO, dP/dt(max) and MAP; Decreased CTPR and LAP; Decreased PRA, aldosterone and BNP; Increased urine volume, uNa^+, uCr and CrCl **Combination therapy:** No increase in HR *vs* control; Intermediate increase in CO; Intermediate decrease in CTPR; Increased dP/dt(max) and MAP; Decreased LAP; Decreased PRA, ALD and BNP; Increased urine volume, uNa^+, uK^+, uCr and CrCl.	59
		mUcn2 infusion through PAC (50 µg + 50µg/h) both isolated and in combination with ACEI captopril (15 mg bolus+ 3mg/h)	**Isolated Ucn-2:** Increased CO, dP/dt(max); Decreased CTPR, LAP and MAP; Decreased PRA, ALD, ET-1, ANP and BNP; Increased urine volume, uNa^+, uK^+, uCr and CrCl **Combination therapy:** Further increase in CO; Further decrease in CTPR and LAP; Further decrease in ALD; Decrease in ET-1, ANP and BNP; Less increase in PRA (*vs* captopril alone) Increased urine volume, uNa^+, uK^+, uCr and CrCl.	64
		mUcn2 infusion through PAC (50 µg + 50µg/h) both isolated and in combination with furosemide (20 mg bolus+3.3 mg/h)	**Isolated Ucn-2:** Increased CO, dP/dt(max); Decreased CTPR, LAP and MAP; Decreased PRA, ALD, AVP, ANP and BNP; Increased urine volume, uNa^+, uK^+, uCr and CrCl **Combination therapy:** Increased CO, dP/dt(max); Decreased CTPR; Further decrease in MAP and LAP; Decreased PRA, ALD, AVP, ANP and BNP; Increased urine volume, uNa^+, uK^+, uCr and CrCl.	65
		mUcn2 infusion through PAC (50 µg + 75µg/h) both isolated and in combination with MRA (200 mg bolus+75 mg/h)	**Isolated Ucn-2:** Increased CO, dP/dt(max); Decreased CTPR and LAP; Decreased PRA, ATII, ALD, AVP, ANP and BNP; Increased plasma cortisol; Increased urine volume, uNa^+, uK^+, uCr and CrCl **Combination therapy:** Increased CO, dP/dt(max); Decreased CTPR and LAP; Decreased PRA, ATII, AVP, ANP and BNP; Initial rise, followed by decreased ALD; Increased plasma cortisol; Increased urine volume, uNa^+, uK^+, uCr and CrCl.	66
	Pacing-induced ADHF	Ucn-2 2-day infusion through PAC (25µg bolus + 0.75 µg/kg/h)	Increased dP/dt(max) and CO; Decreased CVP and LAP; Minor increase in MAP; Decreased PRA, ALD, AVP, ET-1, epinephrine and NE; Increased urine volume, uNa^+, uCr and CrCl Decreased collagen deposition in heart and kidney; Decreased expression of fibrosis, hypertrophy, apoptosis and inflammation markers.	60

ACEI- Angiotensin Converting Enzime Inhibitor; ADHF- Acutely Decompensated Heart Failure; ALD- Aldosterone; ANP- Atrial Natriuretic Peptide; ATII- Angiotensin II; AVP- Arginine Vasopressin; BNP- Brain Natriuretic Peptide; CO- Cardiac Output; Cr- Creatinine; CrCl- Creatinine Clearance; CTPR- Calculated Total Peripheral Resistance; CVP- Central Venous Pressure; ET-1- Endothelin-1; HF- Heart Failure; HR- Heart Rate; IV- intravenous; LAP- Left Atrial Pressure; MAP- Mean Arterial Pressure; MRA- Mineralocorticoid Receptor Antagonist; NE- Norepinephrine; PAC- Pulmonary Artery Catheter; PRA- Plasma Renin Activity; Ucn-1- Urocortin-1; Ucn-2- Urocortin-2; uNa^+-urinary Na^+; uK^+- urinary K^+; uCr- urinary creatinine.

Table I – Pre-clinical studies with Ucn-2 in Heart Failure – Part 2

Species	Model	Posology and route of ministration	Outcomes	Reference
Mouse	MLP-deficiency	IV bolus (7.5 µg/kg)	Increased EF, CO and peak (+) dP/dt; Decreased LV EDV, EDP and ESV; Decreased Tau and peak (-)dP/dt.	29
	MI-induced HF	IV delivery of AAV8.UCn2 (5×10^{11} gc in 100 µl)	Increased EF; Increased +dP/dt and -dP/dt; Decreased Tau; Decreased LV EDD and ESD; No differences in HR, SBP, DBP or MAP; Reduced LV/BW ratio.	61
	WD-induced LV dysfunction	IV delivery of AAV8.UCn2 (2×10^{13} gc/kg)	Improved glucose tolerance; Increased glucose disposal; Decreased fasting glucose; Increased EF, VcFc, EDD and ESD; Increased peak +dP/dt and -dP/dt; Decreased liver fatty infiltration.	63

CO- Cardiac Output; DBP- Diastolic Blood Pressure; EDD- Left Ventricular End-diastolic Diameter; EDP- Left Ventricular End-diastolic Pressure; EDV- Left Ventricular End-diastolic Volume; EF- Ejection Fraction; ESD- Left Ventricular End-systolic diameter; ESV- Left Ventricular End-systolic volume; gc- gene copies; HF- Heart Failure; HR- Heart Rate; IV- intravenous; LV- Left Ventricle; LV/BW- Left Ventricle/Body Weight ratio; MAP- Mean Arterial Pressure; MI- Miocardial Infarction; MLP- muscle-specific LIM protein; SBP- Systolic Blood Pressure; Tau- Mean constant of Ca^{2+} transient decay; Ucn-2- Urocortin-2; Vcfc- velocity of circumferential fiber shortening corrected for heart rate; WD- Western Diet.

Table II – Clinical Trials with Ucn-2 in Heart Failure

Study Design	Patients	Posology and route of ministration	Outcomes	Reference
Single-blind, placebo-controlled, dose-escalation	8 males w/ stable CHF (LVEF≤40%, NYHA class II/III)	1h IV infusion of placebo, 25 and 100µg of hUcn-2 sequentially with a WO period of 2–5 weeks	Increased CO and HR; Decreased SBP, DBP and MAP; Decreased SVR and CW; Higher dose increased LVEF and E; Higher dose decreased LVWMSi; Increased NTproBNP, ACTH, and ADM; Higher dose slightly increased pCr; Slight decrease in urine volume and Na^+excretion.	67
Double-blind, placebo-controlled, randomized	53 patients admitted for ADHF	4h IV infusion of either Ucn-2 (400µg) or placebo as an adjunct to conventional therapy initiated by the physician	Increased CO; Decreased SBP, DBP and CTPR; Decreased pCr, CrCl, urine output and Na^+ excretion; Decreased BNP and NTproBNP; Increased PRA, not accompanied by rises in ALD or ATII.	68
A: randomized, dose-escalation	**A**: 8 HF* patients and 8 gender and age-matched healthy subjects	**A:** Ucn-2 infusions through BA (3.6, 12 and 36 pmol/min) for 6 min with a 30 min WO period	**A:** +60% local vasodilation from baseline in HF patiens; +72% local vasodilation from baseline in healthy subjects;	55
B: double-blind, placebo-controlled, randomized, dose-escalation, crossover study	**B**: 9 HF* patients and 7 healthy subjects *LVEF < 35%, EDD > 5.5 cm, NYHA class II/III	**B:** IV placebo or SNP followed by either Ucn-2 (36, 108, 360 pmol/min) or Ucn-3 for 10 min with 1h WO period	**B:** Increased CI; Decreased MAP and PVRI.	

ACTH- Adrenocorticotrophic hormone; ADHF- Acutely Decompensated Heart Failure; ADM- Adrenomedulin; ALD- Aldosterone; ATII- Angiotensin II; BA- Brachial Artery; BNP- Brain Natriuretic Peptide; CHF- Congestive Heart Failure; CI- Cardiac Index; CO- Cardiac Output; CrCl- Creatinine Clearance; CTPR- Calculated Total Peripheral Resistance; CW- Cardiac Work; DBP- Diastolic Blood Pressure; E- Transmitral early diastolic flow velocity; EDD- Left Ventricular End-diastolic Diameter; HF- Heart Failure; HR- Heart Rate; IV- intravenous; LVEF- Left Ventricle Ejection Fraction; LVWMSi-Left ventricular wall motion score index; MAP: Mean Arterial Pressure; NTproBNP- amino terminal pro-brain natriuretic peptide; NYHA- New York Heart Association; pCr- Plasma creatinine; PRA- Plasma Renin Activity; PVRI- Peripheral Vascular Resistance index; SBP- Systolic Blood Pressure; SVR- Systemic Vascular Resistance; Ucn-2- Urocortin-2; Ucn-3- Urocortin-3; WO-Washout.

Table III – Pre-clinical studies with Ucn-2 in other cardiovascular diseases

Disease	Species	Model	Posology and route of ministration	Outcomes	Reference
Ischemia	Rat	Ischemia/Reperfusion by LAD ligation	IV Ucn-2 (10 µg/kg) before LAD occlusion	Reduced arrhythmias; Approximate 50 % reduction in infarct size.	73
		Ischemia/Reperfusion by LCA ligation	IV Ucn-2 (150 µg/Kg) 5 min. before reperfusion	Increased EF and FS; Decreased ESD and EDD; Reduction in infarct size; Prevention of fibrosis in risk and remote zones.	74
		Ischemia Reperfusion by LAD ligation	IP Ucn-2 (15 µg/Kg) 20 min. before LAD occlusion	Improved contractile function; Reduced necrotic area.	72
	Mouse	MI induced by LCA ligation	SC Ucn-2 (415 µg/Kg/d) for 30 days starting 24h after MI	Increased EF and FS; Decreased ESD and EDD; Decreased HW:BW ratio and LV mass; Reduction in infarct size; Decreased MAP; Increased HR; Decreased Col1 and β-MHC gene expression; Decreased collagen deposition in non-infarcted areas.	75
Systemic Hypertension	Rat	DSS-rat switched to high salt diet at 6 weeks	IP hUcn-2 (2.5 µg/Kg b.i.d) for 5 weeks	Acute and chronic reduction in BP, not accompanied by reflex tachycardia; Prevention of Vcf decrease; Prevention of E/A ratio increase; Decreased PWth and HW/BW ratio.	76
		Spontaneously Hypertensive Rats	IV Ucn-2 (1 µg/kg/d, 3.5 µg/kg/d or 7 µg/kg/d) for 2 weeks	Decreased MAP, LVSP and LVEDP; Increased +dP/dt (max) and -dP/dt (max).	77
Pulmonary Arterial Hypertension	Rat	MCT-induced PAH	IP Ucn-2 (5µg/Kg b.i.d) for 10 days	Increased survival, exercise tolerance and BW; Improved several echocardiographic markers of RV diastolic and systolic functions, dilation and hypertrophy; Improved LV systolic and diastolic functions; Reduced fibrosis and cardiomyocyte CSA; Reduced pulmonary small arteries remodeling; Improved endothelial function.	79
		PAB		Decreased RV hypertrophy and fibrosis; Lessened increase in passive stiffness.	

β-MHC: β Miosin Heavy Chain; BP: Blood Pressure; BW: Body Weight; Col1: Collagen 1; CSA- Cross-sectional Area; DSS- Dahl salt-sensitive; EDD- Left Ventricular End-diastolic Diameter; EF- Ejection Fraction; ESD- Left Ventricular End-systolic diameter; FS- Fractional Shortening; HR: Heart Rate; HW/BW: Heart Weight/Body Weight ratio; IP- intraperitoneal; IV- intravenous; LAD- Left Anterior descending Artery; LCA- Left Coronary Artery; LV- Left Ventricle; LVEDP-left ventricular end diastolic pressure; LVSP-left ventricle systolic pressure; MAP: Mean Arterial Pressure; MCT- Monocrotaline; MI- Miocardial Infarction; PAB- Pulmonary Artery Binding; PAH-Pulmonary Arterial Hypertension; PWth- left ventricle posterior wall thickness; RV- Right Ventricle; SC- subcutaneous; Ucn-2- Urocortin-2; Vcf: velocity of circumferential fiber shortening.

ANEXO 1- Normas da Revista Científica

Revista: Cardiovascular Drugs and Therapy

Editor chefe: Willem J. Remme

ISSN: 1573-7241 (Versão eletrónica)

Fator de impacto (2017): 2.771

Normas da revista

As seguintes normas foram retiradas do *website* da revista (acesso a 19/03/2019): https://www.springer.com/medicine/cardiology/journal/10557

Review Articles

There is no fixed limit on the length of review articles, though concise presentation is expected.

Title Page

The title page should include: The name(s) of the author(s); a concise and informative title; the affiliation(s) and address(es) of the author(s); the e-mail address, and telephone number(s) of the corresponding author and if available, the 16-digit ORCID of the author(s).

Abstract

Please provide an abstract of 150 to 250 words.

Text Formatting

-Normal, plain font (e.g., 10-point Times Roman) for text. Italics for emphasis;

-Automatic page numbering function to number the pages. Do not use field functions;

-Tab stops or other commands for indents, not the space bar;

-Table function, not spreadsheets, to make tables.

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

References

-Reference citations in the text should be identified by numbers in square brackets;

-The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list;

-The entries in the list should be numbered consecutively;

-Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see ISSN.org LTWA. If you are unsure, please use the full journal title;

- For authors using EndNote, Springer provides an output style that supports the formatting of in text citations and reference list (used in this work).

1. Journal article

Smith JJ. The world of science. Am J Sci. 1999;36:234–5.

2. Article by DOI

Slifka MK, Whitton JL. Clinical implications of dysregulated cytokine production. J Mol Med. 2000; https://doi.org/10.1007/s001090000086

3. Book

Blenkinsopp A, Paxton P. Symptoms in the pharmacy: a guide to the management of common illness. 3rd ed. Oxford: Blackwell Science; 1998.

4. Book chapter

Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. In: Bourne GH, Danielli JF, Jeon KW, editors. International review of cytology. London: Academic; 1980. pp. 251–306.

5. Online document

Doe J. Title of subordinate document. In: The dictionary of substances and their effects. Royal Society of Chemistry. 1999. http://www.rsc.org/dose/title of subordinate document. Accessed 15 Jan 1999.

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see ISSN.org LTWA. If you are unsure, please use the full journal title;

For authors

All authors should be mentioned if 6 or less. Only the first 3 followed by et al. if 6 or more.

Tables

-All tables are to be numbered using Arabic numerals;

-Tables should always be cited in text in consecutive numerical order;

-For each table, please supply a table caption (title) explaining the components of the table;

-Identify any previously published material by giving the original source in the form of a reference at the end of the table caption;

-Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

ANEXO 2 – Publicações e apresentações no âmbito do tema abordado na monografia

Durante os três anos que passei enquanto membro de um grupo de investigação no Departamento de Cirurgia e Fisiologia da Faculdade de Medicina, realizei as seguintes apresentações no âmbito do estudo da Urocortina-2 na hipertensão arterial pulmonar:

Monteiro-Pinto C, Adão R, Mendes-Ferreira P, Maia-Rocha C, Santos-Ribeiro D, Pimentel LD, Leite-Moreira AF, Brás-Silva C. Urocortin-2 attenuates experimental pulmonary arterial hypertension and right ventricular dysfunction. IJUP' 17 – 10th Meeting of Young Researchers of University of Porto. 8-10 de fevereiro, 2017; Porto, Portugal.

Monteiro-Pinto C, Adão R, Mendes- Ferreira P, Maia-Rocha C, Santos-Ribeiro D, Pimentel LD, Leite-Moreira AF; Brás-Silva, C. Urocortin-2 improves right ventricular function and attenuates experimental pulmonary arterial hypertension. Yes meeting 2016; 16-18 de setembro, 2016; Porto, Portugal.

Pinto C, Adão R, Mendes-Ferreira P, Maia-Rocha C, Santos-Ribeiro D, Pimentel L, Salgado S, Potus F, Rademaker MT, Bonnet S, Leite-Moreira AF, Brás-Silva C. Urocortin-2 improves right ventricular function and attenuates pulmonary arterial hypertension. Congresso Português de Cardiologia 2017. 22 - 25 de abril, 2017; Albufeira, Portugal.

Fui, ainda, parte integrante do seguinte trabalho de investigação publicado:

Adão R, Mendes-Ferreira P, Santos-Ribeiro D, Maia-Rocha C, Pimentel L, **Monteiro-Pinto C**, Mulvaney EP, Reid HM, Kinsella T, Potus F, Breuils-Bonnet S, Rademaker MT, Provencher S, Bonnet S, Leite-Moreira A, Brás-Silva C. Urocortin-2 improves right ventricular function and attenuates pulmonary arterial hypertension. Cardiovascular Research. 2018;114: 1165–1177.