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Effect of Empagliflozin on Circulating Proteomics in Heart Failure: Mechanistic Insights from the EMPEROR Program

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1 **Effect of Empagliflozin on Circulating Proteomics in Heart Failure: Mechanistic**
2 **Insights from the EMPEROR Program**

3
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44
45 Short tile: Empagliflozin proteomics in heart failure

1 **Abstract**

2 *Background:* Sodium glucose co-transporter 2 (SGLT2) inhibitors improve
3 cardiovascular outcomes in diverse patient populations, but their mechanism of
4 action requires further study.

5 *Aims:* To explore the effect of empagliflozin on circulating levels of intracellular
6 proteins in patients with heart failure, using large-scale proteomics.

7 *Methods:* Over 1250 circulating proteins were measured at baseline, week 12 and
8 week 52 in 1134 patients from EMPEROR-Reduced and EMPEROR-Preserved,
9 using the Olink® Explore 1536 platform. Statistical and bioinformatical analyses
10 identified differentially expressed proteins (empagliflozin vs placebo), which were
11 then linked to demonstrated biological actions in the heart and kidneys.

12 *Results:* At week 12, 32 of 1283 proteins fulfilled our threshold for being differentially
13 expressed, i.e., their levels were changed by $\geq 10\%$ with a false discovery rate $< 1\%$
14 (empagliflozin vs placebo). Among these, nine proteins demonstrated the largest
15 treatment effect of empagliflozin: insulin-like growth factor-binding protein 1,
16 transferrin receptor protein 1, carbonic anhydrase 2, erythropoietin, protein-
17 glutamine gamma-glutamyltransferase 2, thymosin beta-10, U-type mitochondrial
18 creatine kinase, insulin-like growth factor-binding protein 4, and adipocyte fatty acid-
19 binding protein 4. The changes of the proteins from baseline to week 52 were
20 generally concordant with the changes from baseline to week 12, except
21 empagliflozin reduced levels of kidney injury molecule-1 by $\geq 10\%$ at week 52, but not
22 at week 12. The most common biological action of differentially-expressed proteins
23 appeared to be the promotion of autophagic flux in the heart, kidney or endothelium,
24 a feature of 6 proteins. Other effects of differentially-expressed proteins on the heart
25 included the reduction of oxidative stress, inhibition of inflammation and fibrosis, and
26 the enhancement of mitochondrial health and energy, repair and regenerative
27 capacity. The actions of differentially expressed proteins in the kidney involved
28 promotion of autophagy, integrity and regeneration, suppression of renal
29 inflammation and fibrosis, and modulation of renal tubular sodium reabsorption.

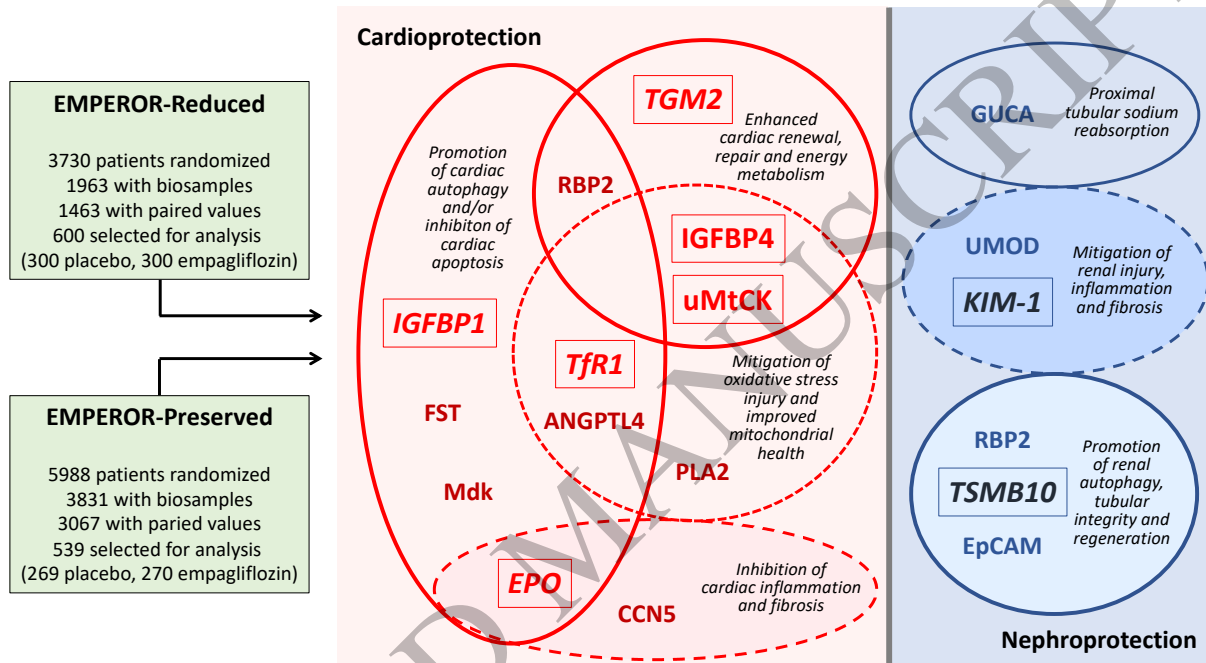
30 *Conclusions:* Changes in circulating protein levels in patients with heart failure are
31 consistent with the findings of experimental studies that have shown that the effects
32 of SGLT2 inhibitors are likely related to actions on the heart and kidney to promote
33 autophagic flux, nutrient deprivation signaling and transmembrane sodium transport.

34 *Key words:* heart failure; proteomics; SGLT2 inhibitors; differentially expressed
35 proteins

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Structural Graphical Abstract

Favorable Biological and Cellular Actions of Differentially Expressed Proteins in the Heart and Kidney



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10 Protective effects on the heart are shown in red; protective effects on the kidney are shown in blue.
 11 Empagliflozin had the largest treatment effect on proteins shown in larger font and in italics. Abbreviations:
 12 IGFBP1, insulin-like growth factor-binding protein 1; TfR1, transferrin receptor protein 1; EPO, erythropoietin;
 13 TGM2, protein-glutamine gamma-glutamyltransferase 2; TMSB10, thymosin beta-10; uMtCK, mitochondrial
 14 creatine kinase U-type; IGFBP4, insulin-like growth factor-binding protein 4; EpCAM, epithelial cell adhesion
 15 molecule; PLA2, phospholipase A2; ANGPTL4, angiotensin-related protein 4; RBP2, retinol-binding protein
 16 162; CCN5, CCN family member 5; ANGPTL4, angiotensin-related protein 4; FST, follistatin; Mdk, midkine;
 17 GUCA, guanylin. RBP2 has both cardioprotective and nephroprotective effects.

18
19
20

1 **Abbreviations**

2	AFABP	adipocyte fatty acid-binding protein 4
3	ANGPTL4	angiopoietin-related protein 4
4	ANGPTL2	angiopoietin-related protein 2
5	CA2	carbonic anhydrase 2
6	CCL16	C-C motif chemokine 16
7	CCL18	C-C motif chemokine 18
8	CCL27	C-C motif chemokine 27
9	CCN5	Connective tissue growth factor/cysteine-rich 61/nephroblastoma
10		overexpressed family member 5
11	CCN5	CCN family member 5
12	CNDP1	beta-Ala-His dipeptidase
13	eGFR	estimated glomerular filtration rate
14	EpCAM	epithelial cell adhesion molecule
15	EPO	erythropoietin
16	FDRq	false discovery rate q-value
17	FST	follistatin
18	GUCA	guanylin
19	IGFBP1	insulin-like growth factor-binding protein 1
20	IGFBP4	insulin-like growth factor-binding protein 4
21	Mdk	midkine
22	NPDC1	neural proliferation differentiation and control protein 1
23	PLA2	phospholipase A2
24	Rarres2	retinoic acid receptor responder protein 2, chemerin
25	RBP2	retinol-binding protein 2
26	SGLT2	sodium-glucose co-transporter 2
27	SRCR	scavenger receptor cysteine-rich domain-containing group B protein
28	SPINK1	serine protease inhibitor Kazal-type 1
29	TfR1	transferrin receptor protein 1
30	TGM2	protein-glutamine gamma-glutamyltransferase 2
31	TMSB10	thymosin beta-10
32	UMOD	uromodulin
33	uMtCK	U-type mitochondrial creatine kinase

34

35

1 Introduction

2

3 Sodium-glucose co-transporter 2 (SGLT2) inhibitors improve cardiovascular
4 outcomes across a wide range of high-risk patients, including those with type 2
5 diabetes, chronic kidney disease, and heart failure with a reduced or preserved
6 ejection fraction.¹ These benefits have been characterized by a decrease in the risk
7 of cardiovascular mortality and heart failure hospitalizations and a reduction in major
8 adverse renal events.²

9

10 The underlying mechanisms by which SGLT2 inhibitors improve heart failure and
11 renal outcomes are incompletely understood. Recent evidence suggests that SGLT2
12 inhibitors may induce a state of starvation mimicry, characterized by enhanced
13 nutrient deprivation signalling and autophagic flux, which leads to an improvement in
14 mitochondrial function, a decrease in oxidative stress, and suppression of
15 proinflammatory and profibrotic pathways.³ It has also been hypothesized that
16 SGLT2 inhibitors may decrease the activity of sodium-hydrogen exchangers in the
17 heart and kidneys, which may reduce intracellular sodium in cardiomyocytes and
18 promote urinary sodium excretion.⁴ Mediation analyses have identified increases in
19 haemoglobin and decreases in uric acid as statistical intermediaries of the benefits of
20 SGLT2 inhibitors on heart failure and renal outcomes.^{5,6} Changes in haemoglobin
21 and uric acid may reflect the effects of enhanced nutrient deprivation signalling on
22 erythropoietin production and the effects of augmented autophagic flux on oxidative
23 stress.^{3,7}

24

25 We performed large-scale proteomic analyses of biosamples collected in the
26 EMPEROR-Reduced and EMPEROR-Preserved trials before and following short-
27 and long-term treatment with empagliflozin to gain insights into the potential
28 mechanisms of action of SGLT2 inhibitors in patients with chronic heart failure.

29

1 **Methods**

3 Study Populations and Biosampling

5 A total of 9718 patients were randomized into the EMPEROR-Reduced (n=3730)
6 and EMPEROR-Preserved (n=5988) trials. Participation in sampling for biobanking
7 of plasma, serum, DNA, and urine was voluntary and not a prerequisite for
8 participation in the trials. Biosamples were collected only if a separate informed
9 consent had been signed, in accordance with local ethical and regulatory
10 requirements. The countries that participated in the biobanking substudy and the
11 methodology of sample collection are described in *Supplementary Methods 1*.

13 In EMPEROR-Reduced, 3831 patients gave consent for the collection of biosamples,
14 and of these, 1963 patients had samples available at baseline (i.e., before
15 randomization) and at one or both on-treatment sampling visits (at week 12 or week
16 52). In EMPEROR-Preserved, of the 3067 patients who gave consent for the
17 collection of biosamples, 1463 patients had samples available at baseline and at one
18 or both on-treatment sampling visits (at week 12 or week 52). The plasma samples
19 of 600 patients (out of 1963) in EMPEROR-Reduced and of 539 (out of 1463) in
20 EMPEROR-Preserved were randomly selected, and patients were stratified by
21 treatment assignment and data availability. This sample size was determined by
22 budgetary constraints. A total of 599 patients (out of the 600) in EMPEROR-
23 Reduced and 535 (out of 539) in EMPEROR-Preserved had good quality samples
24 with available data both at baseline and at one or both post-randomization visits;
25 thus, a total of 1134 patients were included in the pooled analysis (EMPEROR-
26 Pooled), see *Supplementary Methods 2*.

28 In these 1134 patients, measurements of circulating proteins were performed using
29 the Olink® Explore 1536 platform, which utilizes proximity extension assay (PEA)
30 technology with a dual-recognition DNA-coupled readout, in which oligonucleotide-

1 labelled antibody probe pairs bind to their respective targets.⁸ The Olink[®] Explore
2 1536 platform consists of 1472 protein assays and 64 internal controls, and divided
3 into four 384-plex panels, and in each panel, overlapping assays of interleukin-6,
4 interleukin-8, and tumor necrosis factor- α are included as an additional quality
5 control. The platform provides \log_2 normalized protein expression values with relative
6 quantification. Performance of the assays was blinded to the treatment allocation.
7 Samples were allocated in a random order to avoid any systematic influence of study
8 or timepoint. Protein expression values were missing when samples failed quality
9 control (as specified by the manufacturer), and missing values were not imputed.
10 Proteins that were below the limit of detection in >33% of baseline samples were
11 excluded from the analysis. A total of 1283 proteins were evaluated.

13 Statistical and Bioinformatical Analysis

14
15 Differences in the level of proteins were assessed using mixed models for repeated
16 measurements, in which we calculated the between-group difference (empagliflozin
17 vs placebo) in the change in plasma concentrations for each protein from baseline to
18 week 12 and week 52, while adjusting for the baseline covariates that had been
19 prespecified in the statistical plan for each trial (i.e., age, sex, geographical region,
20 diabetes, left ventricular ejection fraction, estimated glomerular filtration rate
21 [eGFR]). We also prespecified an adjustment for the baseline protein expression
22 and trial assignment (EMPEROR-Reduced and EMPEROR-Preserved). The change
23 from baseline in eGFR was included as a covariate in a post hoc sensitivity analysis.

24
25 We identified differentially-expressed proteins as those fulfilling two independent
26 criteria: (1) proteins with $|\log_2 \text{fold change}| > \log_2(1.1)$, corresponding to a $\geq 10\%$
27 increase from baseline ($\log_2 \text{fold change} > 0.1375$) or a symmetric $> 9.09\%$ decrease
28 from baseline; and (2) a false discovery rate q-value (FDR_q) $< 1\%$, which applies a
29 Benjamini-Hochberg correction to the raw P-value, in order to minimize inflation of a

1 false positive error rate due to multiplicity of comparisons.⁹ Significance of
2 differences between treatment effects at week 12 and 52 were investigated by a
3 treatment-by-visit interaction term in the model.

4
5 All proteins measured in the Olink[®] platform are known to exert diverse function
6 across many biological domains. We initially grouped differentially-expressed
7 proteins according to their reported actions in oncology, but these groupings had
8 little meaning in cardiology and nephrology. Therefore, we performed an extensive
9 biomedical literature search to identify the biological effects of differentially-
10 expressed proteins in the heart and kidneys.

11

12 **Results**

13

14 Patient Characteristics

15

16 The 1134 patients had a mean age of 70 years, 32% were women, 85% were White,
17 and 57% were recruited in Europe. In addition, 48% had type 2 diabetes, the mean
18 left ventricular ejection fraction was 40%, and the mean eGFR was 61 ml/min/1.73
19 m². When the 1134 patients whose biosamples were analysed were compared with
20 the 8584 patients who were randomized in the EMPEROR trial but were not
21 represented in the current analyses, patients not in the current analysis were more
22 likely to be women (37% vs 32%), less likely to be White (72% vs 85%), or from
23 Europe (40% vs 57%), and had a higher ejection fraction (45% vs 40%),

24 *Supplementary Table 1.*

25

26

1 Differentially Expressed Proteins at Week 12

2
3 Based on our prespecified criteria and covariate adjustment, 32 of 1283 proteins
4 fulfilled our threshold for being differentially expressed at week 12; all were
5 increased by $\geq 10\%$ (\log_2 fold change > 0.1375) and had a false discovery rate $< 1\%$
6 (corresponding generally to a raw P-value < 0.0007 for the difference between
7 empagliflozin and placebo), Table 1.

8
9 Among these, nine proteins demonstrated the largest treatment effect of
10 empagliflozin (\log_2 fold change > 0.201 (corresponding to a $> 15\%$ increase with a
11 false discovery rate $< 1\%$). These were insulin-like growth factor-binding protein 1
12 (IGFBP1), transferrin receptor protein 1 (TfR1), carbonic anhydrase 2 (CA2),
13 erythropoietin (EPO), protein-glutamine gamma-glutamyltransferase 2 (TGM2),
14 thymosin beta-10 (TMSB10), mitochondrial creatine kinase U-type (uMtCK), insulin-
15 like growth factor-binding protein 4 (IGFBP4), and adipocyte fatty acid-binding
16 protein 4 (AFABP4). Among these, the effect of empagliflozin on TfR1 was
17 particularly noteworthy with a nearly 20% increase and an exceptionally low false
18 discovery rate (2.5×10^{-10}) with a raw P-value of 1.9×10^{-13} .

19
20 The effects of empagliflozin (corrected for placebo) on all measured circulating
21 proteins (corrected for placebo) from baseline to week 12 are shown in
22 *Supplementary Table 2* and displayed in *Supplementary Figure 1*. No differentially
23 expressed protein showed a statistically significant interaction between EMPEROR-
24 Reduced and EMPEROR-Preserved at week 12, *Supplementary Table 3*. No
25 differentially expressed protein met the criteria for a meaningful decrease in level at
26 week 12. Of note, N-terminal prohormone B-type natriuretic peptide declined at 12
27 weeks, but the change was not statistically significant.

28

1 In a post hoc sensitivity analysis, we examined the effects of empagliflozin vs
2 placebo on the changes in protein levels from baseline for all 1283 proteins after the
3 inclusion of the change in eGFR from baseline to week 12 as an additional covariate.
4 No new proteins were found to be differentially expressed. Of the 32 differentially
5 expressed proteins in our original model, adjustment for the change in eGFR did not
6 meaningfully influence the effect size for proteins with the largest treatment effect,
7 but 5 of the 6 proteins with the smallest changes in our original model now fell
8 meaningfully below the threshold of \log_2 fold change of 0.1375 following adjustment
9 (Table 1). For the remaining 27 proteins, the false discovery rate generally
10 increased modestly, but remained $< 5\%$ for all proteins, and was $< 2\%$ for all but 4
11 proteins.

12 13 Differentially Expressed Proteins at Week 52

14
15 The changes of the proteins from baseline to week 52 were generally concordant
16 with the changes from baseline to week 12 (Supplementary Figure 1), as
17 demonstrated by a lack of significant treatment-by-time interactions for all proteins,
18 with one exception. When compared with placebo, empagliflozin did not have a
19 meaningful effect on kidney injury molecule-1 (KIM-1) at week 12, but it decreased
20 KIM-1 at week 52 (Figure 1), \log_2 fold change -0.18 (95% CI -0.10 to -0.27), a 12%
21 decrease with a false discovery rate of 0.006%. The effect of empagliflozin on KIM-1
22 at 52 weeks was significantly different from that at 12 weeks, visit-by-treatment
23 interaction false discovery rate of 0.008%.

24 25 Biological Actions of Differential Expressed Proteins on the Heart and Kidney

26
27
28 As noted above, after adjustment for changes in eGFR, the circulating levels of 27
29 proteins were increased by $\approx 10\%$ or more by empagliflozin at 12 weeks, and one

1 protein was decreased by at least 10% by the drug at 52 weeks. The known
2 biological effects of these 28 proteins are shown in Table 2.¹⁰⁻⁶²

3
4 Fourteen proteins have established effects in the heart, and of these, the most
5 common effect was the promotion of autophagic flux (5 proteins), which was
6 particularly characteristic of the three cardiac-acting proteins that had the largest
7 effect size (IGFBP1, TfR1 and EPO), Table 1.^{10,13,15} Most of the other 11 proteins
8 have been shown to reduce oxidative stress or its consequences, inhibit apoptosis,
9 inflammation and fibrosis, and enhance the energy, repair and regenerative capacity
10 of the heart (Table 2 and Figure 2).

11
12 Nine proteins have established effects in the kidney (Table 2), and of these, three
13 proteins (carbonic anhydrase 2, guanylin and uromodulin) have actions on sodium
14 transport in the renal tubules.^{46,47,50} Two proteins have been shown to inhibit renal
15 injury, inflammation, and fibrosis (uromodulin and KIM-1),^{51,54} and three proteins play
16 a role in promoting renal autophagy, tubular integrity and regeneration (RBP2,
17 TSMB10 and EpCAM), Figure 2 and Table 2.^{24,56,57}

18

19 **Discussion**

20 Our proteomics analysis of blood samples taken from participants pooled from the
21 EMPEROR-Reduced and EMPEROR-Preserved trials indicate that circulating levels
22 of approximately 30 proteins changed meaningfully during SGLT2 inhibition with
23 empagliflozin (Structured Graphical Abstract). Concentrations of these proteins
24 changed by at least 10%, and they did so with substantial consistency, yielding
25 results that were highly statistically significant, even after major adjustment for
26 multiplicity of testing. Our thresholds for identifying differentially expressed proteins
27 were similar to or more stringent than those used in other cardiovascular proteomics
28 analyses; we used a false discovery rate of < 1%, rather than the < 5% that has

1 been commonly used in other studies.⁶³ Importantly, the changes in the circulating
2 levels of most differentially-expressed proteins were not related to changes in renal
3 clearance than may have been produced by empagliflozin as a result of its action to
4 reduce intraglomerular filtration pressures, since our results did not meaningfully
5 change following adjustment for changes in glomerular filtration rate. It should be
6 understood that most of the proteins that we assessed act intracellularly, and it is
7 plausible that changes in circulating levels parallel changes in the intracellular
8 concentration of these proteins with subsequent release from cytosol. However, the
9 magnitude of this release does not necessarily reflect the intracellular expression or
10 activation of these proteins, whose stoichiometric relationships may not be linear.
11 Furthermore, our proteomics analyses cannot identify the organ sources of this
12 protein release.

13
14 In experimental studies, SGLT2 inhibitors have been shown to exert cardioprotective
15 and nephroprotective effects by promoting autophagic flux and mitigating apoptosis,
16 muting oxidative and other cellular stresses, inhibiting proinflammatory and
17 profibrotic pathways, and enhancing cellular energy stores and metabolism.^{3,64-68} It
18 is therefore noteworthy that most of the 33 differentially expressed proteins identified
19 in our proteomics analyses have been shown to exert favourable effects on the heart
20 and kidney in hypothesis-driven experimental studies performed in animal models of
21 cardiac and renal injury (Table 2). Of particular note, six proteins have been shown
22 to promote autophagic flux in the heart, kidney or endothelium, i.e., IGFBP1, TfR1,
23 EPO, follistatin, ANGPTL4 and RBP2.^{10,11,13,15,19, 24,30}

24
25 Five proteins have been shown to reduce the levels or consequences of oxidative
26 stress and improve mitochondrial health in the myocardium (TfR1, PLA2,
27 ANGLPTL4, IGFBP4 and uMtCK).^{13,28,29,31,36} Four proteins have been reported to
28 enhance energy metabolism, cardiac repair or the renewal of cardiomyocytes
29 (RBP2, IGFBP4, TGM2 and uMtCK).^{23,32-35} As a result of these and other actions,

1 many of the differentially-expressed proteins have favourable effects to retard
2 maladaptive ventricular hypertrophy or remodelling, Table 2. In addition, two
3 proteins have demonstrated effects to mitigate renal injury and inflammation
4 (uromodulin and KIM-1);^{49,52} three proteins have been shown to promote renal
5 autophagy or enhance the integrity or regeneration of renal tubular cells (RBP2,
6 TSMB10 and EpCAM),^{55,57} and three proteins (carbonic anhydrase 2, guanylin and
7 uromodulin) play a role in renal tubular sodium transport.^{47,49} Therefore, our
8 proteomics analyses suggest that the pathways that are influenced by SGLT2
9 inhibitors in patients with heart failure are similar to those influenced by these drugs
10 in animal models of cardiac or renal stress.⁶⁴⁻⁶⁹

11
12 Importantly, our conclusions remained unaltered even when we focused on proteins
13 with the most marked changes, Figure 1. The protein that showed the most striking
14 increase was IGFBP1, which binds to insulin-like growth factor 1 (IGF1) to limit its
15 activity. IGF1 has demonstrable deleterious effects on the heart to suppress
16 autophagy, and its upregulation promotes the development of cardiomyopathy.¹⁰
17 The protein that showed the second largest treatment effect in the current study was
18 TfR1, which is required for iron transport into cardiomyocytes to prevent contractile
19 dysfunction,⁷⁰ but it also enhances nutrient deprivation signaling.¹⁴ Knockout of TfR1
20 leads to mitochondrial derangements, suppression of autophagy and the
21 development of cardiomyopathy.¹³ The third most differentially expressed protein
22 related to heart function was EPO, which is classically known as the principal
23 stimulus to red blood cell production. However, EPO also exerts direct effects on the
24 heart (independent of its actions on erythrocytosis) to promote autophagy, prevent
25 adverse ventricular remodelling, and mute proinflammatory and profibrotic signaling
26 in the myocardium.¹⁵⁻¹⁷ Interestingly, all three proteins are functionally related to the
27 increases in haemoglobin seen in clinical trials with SGLT2 inhibitors, a physiological
28 change that is the most important statistical mediator of the benefit of SGLT2
29 inhibitors to reduce heart failure and major adverse renal events.^{5,6} Specifically,

1 IGFBP1 promotes the stability of hypoxic inducible factor 1- α ,¹¹ an important
2 endogenous stimulus to the production of erythropoietin, while TfR1 ensures the
3 adequacy of intracellular levels of iron in erythrocytes.⁷¹ Interestingly, increased
4 levels of ANGPTL2 may also serve as an indicator of increased hypoxic inducible
5 factor 1- α signaling.⁶²

6
7 Three prior studies have reported the results of proteomics analyses before and after
8 SGLT2 inhibition, but the three studies each evaluated fewer than 80 patients, less
9 than 10% of the patients in the current EMPEROR proteomics substudy.^{63,72,73} Of the
10 three, the study most comparable to our analyses was carried out by Ferrannini et
11 al,⁶³ although that study differed from ours in several important respects. First,
12 Ferrannini et al studied the effects of empagliflozin in patients with type 2 diabetes,
13 whereas we studied the effects of empagliflozin in patients with chronic heart failure.
14 Ferrannini et al measured proteins using an aptamer-based assay, whereas we
15 performed measurements using a proximity extension assay method. Ferrannini et
16 al performed measurements after only 4 weeks of treatment, whereas we carried out
17 our assessments after 12 and 52 weeks of therapy. Despite these differences, it is
18 noteworthy that Ferrannini et al. also observed that the proteins most prominently
19 affected by SGLT2 inhibition were IGFBP1, TfR1, EPO and adipocyte fatty acid
20 binding protein 4 (AFABP4), findings that are strikingly concordant with the major
21 observations of the current proteomics analyses from the EMPEROR trials.
22 Interestingly, IGFBP1, TfR1, EPO and AFABP4 are all linked to increased activity of
23 sirtuin-1, a nutrient deprivation sensor that promotes autophagy and is known to be
24 increased by SGLT2 inhibitors in experimental studies and that appears to be
25 essential to mediating their benefits.⁷⁴ Experimentally, the effects of SGLT2
26 inhibitors to reduce cellular stress and attenuate apoptosis in cardiomyocytes are
27 abolished when the actions of sirtuin-1 are inhibited.⁷⁵ It is therefore noteworthy that
28 sirtuin-1 directly stimulates the expression of both IGFBP1 and AFABP4;^{12,42} TfR1 is

1 required for sirtuin-1 synthesis;¹⁴ and sirtuin-1 can stimulate the production of EPO
2 by an action on hypoxia-inducible factor 2- α .¹⁸

3
4 Our proteomics findings are also consistent with the upregulation of proteins that
5 have favourable effects on renal structure and function. Upregulation of RBP2 may
6 enhance the ability of retinoic acid to prevent renal injury by promoting
7 autophagic flux in renal parenchymal cells.²⁴ Increases in circulating levels of
8 TSAMB10 and EpCAM may be critical to establishment of the adhesion and polarity
9 of renal tubular cells, a critical step in their regeneration following injury.⁵⁵⁻⁵⁸ During
10 long-term treatment, SGLT2 inhibition produced meaningful decreases in KIM-1,
11 which mediates renal tubular cell injury and apoptosis and promotes tubulointerstitial
12 inflammation and fibrosis.⁵²⁻⁵⁴ The concerted actions of these differentially-increased
13 proteins closely mimic the known effects of SGLT2 inhibitors to prevent renal injury,
14 inflammation, and fibrosis in experimental studies of renal stress in animal models.⁶⁴

15
16 Interestingly, several of our differentially-expressed proteins may represent a
17 mechanism by which the kidney may compensate for the renal tubular actions of
18 SGLT2 inhibitors. SGLT2 inhibitors interfere with sodium reabsorption in the
19 proximal renal tubule through an action to inhibit both SGLT2 and sodium-hydrogen
20 exchanger isoform 3 (NHE3).^{69,76} Guanylin also acts in the proximal tubule to inhibit
21 NHE3,⁴⁷ but it is secreted following a dietary salt load;⁷⁷ its action reinforces the
22 effect of SGLT2 inhibitors to promote the urinary excretion of sodium and
23 bicarbonate.^{76,78} Yet, the observed increase in levels of CA2 may represent a
24 compensatory mechanism to these effects. CA2 binds to NHE3 to increase its
25 activity,⁴⁶ and CA2 enhances the renal tubular adaptation to the increase in urinary
26 bicarbonate that follows the action of SGLT2 inhibitors and guanylin to inhibit
27 NHE3.⁷⁹ In addition, CA2 acts to counter the increase in free water clearance that is
28 seen when glycosuria is induced by SGLT2 inhibitors.⁸⁰ Uromodulin can further
29 counteract the natriuretic effect of SGLT2 inhibitors by enhancing sodium

1 reabsorption more distally in the nephron.⁵⁰ The activation of these
2 counterregulatory mechanisms may explain why the initial natriuretic and osmotic
3 diuretic response to SGLT2 inhibitors is not sustained.⁸¹⁻⁸³ Accordingly, we observed
4 no meaningful decrease in NT-proBNP in the current study, a finding consistent with
5 our earlier observations based on a conventional ELISA assay.⁸⁴

6
7 Our findings should be considered in light of certain strengths and limitations. Our
8 proteomic analyses examined >1200 proteins in blood collected from >1100 patients
9 who participated in two placebo-controlled randomized trials. Therefore, our study
10 represents the largest proteomic database to date on the effects of SGLT2 inhibitors.
11 Despite this advantage, we had insufficient number of patients in each trial to
12 adequately examine potential differences in the effects of SGLT2 inhibitors between
13 patients with a reduced and preserved ejection fraction, and we needed to pool our
14 data across both trials to maximize statistical power. Another strength of the study is
15 that we assessed changes at both 12 and 52 weeks, enabling us to show that the
16 effects seen early in treatment were generally not meaningfully different during long-
17 term therapy. Although we adjusted for changes in renal function that occurred
18 during the first 12 weeks, we did not adjust for changes in concomitant medications,
19 which have the potential to influence protein expression independent of the study
20 medication; however, changes in background therapy during the first 12 weeks were
21 modest.

22
23 Most importantly, although our proximal extension assay has certain advantages
24 over aptamer-based methods (which measure protein fragments), our Olink platform
25 measured only a small fraction of the intracellular proteins that have been implicated
26 in the action of SGLT2 inhibitors in experimental studies. Furthermore, we did not
27 measure many of the proteins that Ferrannini et al⁶³ noted to be meaningfully
28 changed by empagliflozin using aptamer-based methods (including growth
29 differentiation factor 15 and ferritin). Most importantly, we did not directly measure

1 any of the proteins that are involved in enhanced nutrient deprivation signalling and
2 have been implicated in mediating the actions of SGLT2 inhibitors on
3 cardiomyocytes and renal tubular cells (e.g., sirtuin-1, proliferator-activated receptor
4 gamma coactivator 1- α , phosphorylated mammalian target of rapamycin, and
5 phosphorylated AMP-activated protein kinase). These should be the focus of further
6 investigation.

7

8 **Conclusion**

9 Proteomics is rapidly emerging as an innovative approach to gaining potential
10 insights into the mechanisms of disease and drug action. Our proteomics analysis of
11 blood samples taken from participants pooled from two trials of empagliflozin in heart
12 failure identified differential expression of a small select group of circulating proteins
13 following SGLT2 inhibition. The biological effects of differentially-expressed proteins
14 on the heart were primarily focused on the promotion of autophagic flux, but they
15 also included favorable effects to reduce oxidative stress, inhibit inflammation and
16 fibrosis, and enhance the energy stores and the repair and regenerative capacity of
17 the heart. The effects of differentially-expressed proteins in the kidney involved the
18 enhancement of renal autophagy, suppression of renal inflammation and fibrosis,
19 and promotion of renal tubular integrity and regeneration as well as compensatory
20 mechanisms that can limit the ability of SGLT2 inhibitors to produce sustained
21 increases in sodium and water excretion. The actions of differentially-expressed
22 proteins identified in patients with heart failure are consistent with the findings of
23 experimental studies that have linked the benefits of SGLT2 inhibitors on the heart
24 and kidney to their actions on autophagy, inflammation and fibrosis, and cellular
25 stress and viability. Our findings suggest that the results of these experimental
26 studies are likely to be highly relevant to the clinical setting.

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Data sharing

To ensure independent interpretation of clinical study results and enable authors to fulfil their role and obligations under the ICMJE criteria, Boehringer Ingelheim grants all external authors access to clinical study data pertinent to the development of the publication. In adherence with the Boehringer Ingelheim Policy on Transparency and Publication of Clinical Study Data, scientific and medical researchers can request access to clinical study data when it becomes available on Vivli - Center for Global Clinical Research Data, and earliest after publication of the primary manuscript in a peer-reviewed journal, regulatory activities are complete, and other criteria are met. Please visit Medical & Clinical Trials | Clinical Research | MyStudyWindow for further information.

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19

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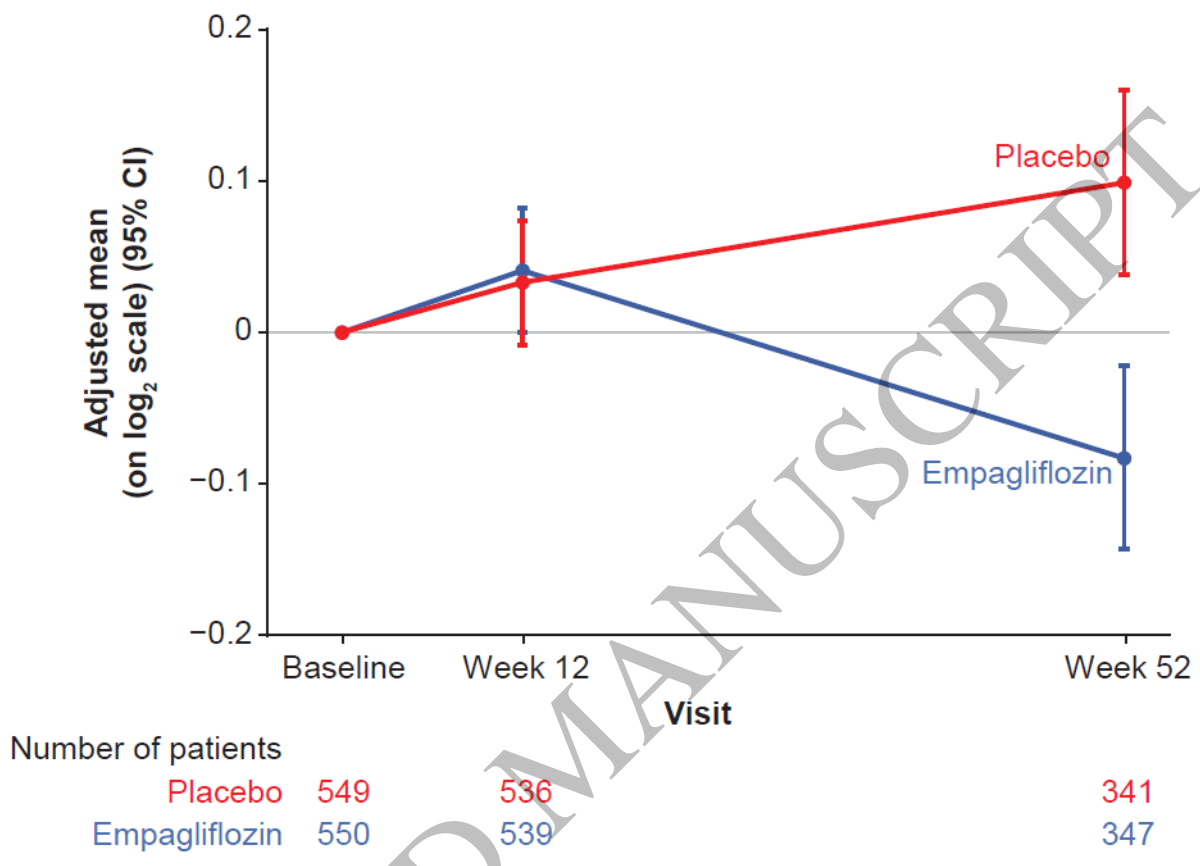
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ACCEPTED MANUSCRIPT

1 **Figure 1. Kidney Injury Molecule-1 (KIM-1): Changes from Baseline in the**
2 **Empagliflozin and Placebo Groups at Week 12 and Week 52**
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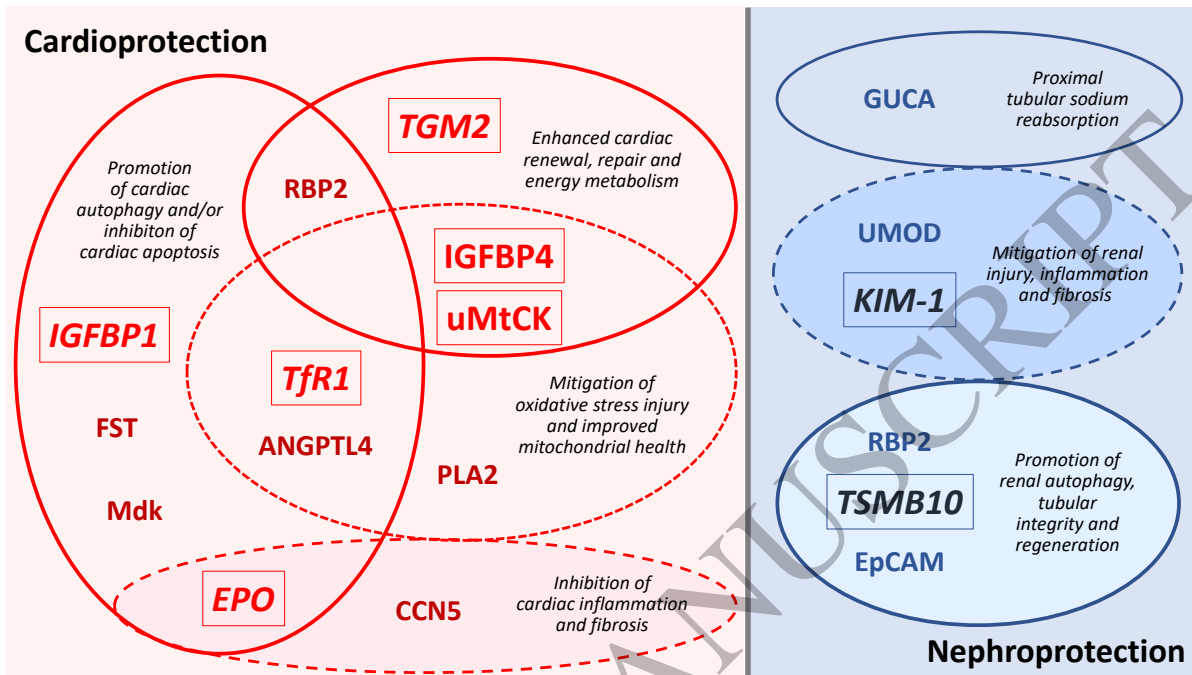


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ACCEPTED MANUSCRIPT

1 **Figure 2. Favorable Biological and Cellular Actions of Differentially Expressed**
 2 **Proteins in the Heart and Kidney**

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9 **Legend**

10 Protective effects on the heart are shown in red; protective effects on the kidney are shown in
 11 blue. Empagliflozin had the largest treatment effect on proteins shown in larger font and in
 12 italics. Abbreviations: IGFBP1, insulin-like growth factor-binding protein 1; TfR1, transferrin
 13 receptor protein 1; EPO, erythropoietin; TGM2, protein-glutamine gamma-glutamyltransferase 2;
 14 TMSB10, thymosin beta-10; uMtCK, mitochondrial creatine kinase U-type; IGFBP4, insulin-like
 15 growth factor-binding protein 4; EpCAM, epithelial cell adhesion molecule; PLA2, phospholipase
 16 A2; ANGPTL4, angiotensin-related protein 4; RBP2, retinol-binding protein 2; CCN5, CCN
 17 family member 5; CCL16, C-C motif chemokine 16; NPDC1, neural proliferation differentiation
 18 and control protein 1; ANGPTL4, angiotensin-related protein 4; FST, follistatin; Mdk, midkine;
 19 GUCA, guanylin. RBP2 has both cardioprotective and nephroprotective effects.

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Table 1. Differential Expressed Proteins (Empagliflozin vs Placebo) at Week 12

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Protein	UniprotID	Before adjustment for change in eGFR		After adjustment for change in eGFR	
		Empagliflozin vs placebo, log ₂ fold change (95%CI)	False discovery rate (%)	Empagliflozin vs placebo, log ₂ fold change (95%CI)	False discovery rate (%)
IGFBP1	P08833	0.27 (0.14-0.40)	0.16	0.25 (0.13-0.38)	0.55
TfR1	P02786	0.26 (0.19-0.32)	<0.0001	0.25 (0.18-0.32)	<0.001
CA2	P00918	0.25 (0.11-0.39)	0.81	0.26 (0.12-0.40)	1.02
EPO	P01588	0.25 (0.11-0.38)	0.65	0.24 (0.10-0.37)	1.53
TGM2	P21980	0.25 (0.11-0.38)	0.60	0.25 (0.12-0.38)	1.02
TMSB10	P63313	0.22 (0.11-0.34)	0.43	0.19 (0.08-0.31)	2.18
uMtCK	P12532	0.21 (0.10-0.32)	0.43	0.22 (0.11-0.33)	0.69
IGFBP4	P22692	0.21 (0.13-0.30)	0.01	0.17 (0.09-0.25)	0.31
AFABP4	P15090	0.21 (0.12-0.29)	0.02	0.17 (0.09-0.25)	0.31
CCL18	P55774	0.20 (0.11-0.29)	0.08	0.19 (0.10-0.28)	0.31
Follistatin	P19883	0.20 (0.13-0.26)	<0.0001	0.19 (0.12-0.26)	<0.0001
Midkine	P21741	0.20 (0.11-0.29)	0.14	0.18 (0.09-0.27)	0.55
Guanylin	Q02747	0.20 (0.13-0.26)	<0.0001	0.17 (0.11-0.23)	<0.001
SPINK1	P00995	0.20 (0.13-0.26)	<0.01	0.16 (0.10-0.23)	0.02
Rarres2	Q99969	0.19 (0.10-0.29)	0.18	0.17 (0.08-0.27)	0.96
CNDP1	Q96KN2	0.19 (0.11-0.28)	0.07	0.19 (0.11-0.27)	0.19
EpCAM	P16422	0.19 (0.10-0.28)	0.17	0.20 (0.10-0.29)	0.29
CCL27	Q9Y4X3	0.19 (0.09-0.29)	0.61	0.17 (0.07-0.27)	2.16
SRCR	Q8WTU2	0.18 (0.09-0.27)	0.21	0.18 (0.09-0.27)	0.48
PLA2	P14555	0.18 (0.08-0.27)	0.54	0.16 (0.07-0.26)	2.07
Promotilin	P12872	0.18 (0.09-0.26)	0.26	0.16 (0.08-0.25)	1.02
ANGPTL4	Q9BY76	0.17 (0.11-0.23)	0.00	0.16 (0.09-0.22)	0.01
RBP2	P50120	0.17 (0.07-0.26)	0.98	0.15 (0.05-0.24)	4.02
Uromodulin	P07911	0.16 (0.10-0.22)	<0.0001	0.18 (0.12-0.23)	<0.0001
Cystatin-F	Q76096	0.16 (0.07-0.25)	0.63	0.15 (0.06-0.24)	1.89
CCN5	Q76076	0.15 (0.08-0.22)	0.14	0.14 (0.07-0.21)	0.69
CCL16	Q15467	0.15 (0.08-0.22)	0.19	0.13 (0.06-0.21)	1.02
Osteopontin	P10451	0.14 (0.08-0.21)	0.18	0.13 (0.06-0.20)	1.02
ANGPTL2	Q9UKU9	0.14 (0.07-0.22)	0.54	0.14 (0.06-0.21)	1.16
NPDC1	Q9NQX5	0.14 (0.08-0.21)	0.07	0.11 (0.06-0.17)	1.02
Elafin	P19957	0.14 (0.07-0.21)	0.44	0.11 (0.04-0.18)	3.96
Cystatin-M	Q15828	0.14 (0.08-0.20)	0.10	0.11 (0.05-0.17)	1.02

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4Legend: IGFBP1, insulin-like growth factor-binding protein 1; TfR1, transferrin receptor protein 1; CA2, carbonic anhydrase 2; EPO, erythropoietin; TGM2, protein-glutamine gamma-glutamyltransferase 2; TMSB10, thymosin beta-610; uMtCK, creatine kinase U-type, mitochondrial; IGFBP4, insulin-like growth factor-binding protein 4; AFABP, adipocyte fatty acid-binding protein 4; CCL18, C-C motif chemokine 18; SPINK1, serine protease inhibitor Kazal-type 81; Rarres2, retinoic acid receptor responder protein 2; CNDP1, beta-Ala-His dipeptidase; EpCAM, epithelial cell adhesion molecule; CCL27, C-C motif chemokine 27; SRCR, scavenger receptor cysteine-rich domain-containing group B protein; PLA2, phospholipase A2; ANGPTL4, angiotensinogen-related protein 4; RBP2, retinol-binding protein 2; CCN5, CCN family member 5; CCL16, C-C motif chemokine 16; NPDC1, neural proliferation differentiation and control protein 1; ANGPTL2, angiotensinogen-related protein 2.

Table 2. Biological Effect of Differentially Expressed Proteins on Heart and Kidney

Protein	Cellular Action	Effects on Heart, Kidney and Other Sites
<i>Proteins With Effects on the Heart</i>		
Insulin-like growth factor-binding protein 1 (IGFBP1)	Promotion of cardiac autophagy	IGF1 promotes heart failure by inhibiting cardiac autophagy; increases in IGFBP1 interfere with actions of IGF1, thus promoting autophagy. ¹⁰ IGFBP1 also promotes HIF-1 α stability and inhibits cardiomyocyte apoptosis. ¹¹ Expression of IGFBP1 is upregulated by sirtuin-1. ¹²
Transferrin receptor protein 1 (TfR1)	Promotion of cardiac iron metabolism and cardiac autophagy; improved mitochondrial health	Required for iron transport into cardiomyocytes. TfR1 knockout leads to mitochondrial dysfunction, down-regulation of cardiac autophagy proteins and cardiomyopathy, which can be prevented by sirtuin-1 activation. ¹³ TfR1-mediated changes in transmembrane electron transport provides NAD ⁺ required for sirtuin activation. ¹⁴
Erythropoietin (EPO)	Promotion of cardiac autophagy and inhibition of cardiac fibrosis	Prevents cardiac remodeling, resulting from promotion of autophagy and mitigates apoptosis and inflammation and fibrosis in the heart. ¹⁵⁻¹⁷ Expression and actions are AMPK- and sirtuin-dependent. ^{15,17,18}
Follistatin (FST)	Promotion of cardiac autophagy	Follistatin and follistatin-like proteins interact with the same receptors. Follistatin-like protein 1 reduces myocardial injury, apoptosis, remodeling, hypertrophy and fibrosis by promoting autophagy through effects on AMPK. ^{19,20}
Retinol-binding protein 2 (RBP2)	Promotion of renal autophagy; inhibition of cardiac apoptosis and enhanced cardiac regenerative capacity	Retinoic acid levels decline in heart failure. Facilitates dietary retinol uptake, thereby mitigating cardiomyocyte apoptosis and promoting cardiac regenerative capacity. ²¹⁻²³ Prevents renal injury by promoting autophagy in the kidney. ²⁴
Midkine (Mdk)	Inhibition of cardiac apoptosis	Reduces cardiac injury, mitigation of cardiac apoptosis and remodeling; promotes angiogenesis. ^{25,26}
Phospholipase A2, membrane-associated (PLA2)	Promotion of cardiac repair following oxidative stress	Calcium-independent PLA2 in ventricular cardiomyocytes localizes to mitochondria and peroxisomes and acts as a phospholipid repair enzyme following oxidative damage. Inhibition of phospholipase A2 is a mechanism underlying anthracycline cardiotoxicity. ^{27,28}
Angiotensin-related protein 4 (ANGPTL4)	Reduction of oxidative stress and promotion of endothelial cell autophagy	Inhibits lipoprotein lipase in cardiomyocytes. Prevents fatty acid-induced oxidative stress. ²⁹ Preserves endothelial integrity by promotion of autophagy, thereby supporting myocardial function. ³⁰

Insulin-like growth factor-binding protein 4 (IGFBP4)	Reduction of oxidative stress and promotion of cardiac regenerative capacity	Produces cardioprotection by minimizing the DNA injury produced by oxidative stress and promotes angiogenesis. ³¹ Enhances induction of cardiomyocytes from pluripotential stem cells by inhibition of the Wnt/ β -catenin signaling, independent of its binding to IGF. ³²
Protein-glutamine gamma-glutamyl-transferase 2 (TGM2)	Enhanced cardiac ATP synthesis, cardiac repair and regenerative capacity	Enhanced fatty acid utilization and ATP synthesis. ³³ Promotes cardiac repair and regenerative capacity. ³⁴
Mitochondrial creatine kinase, U-type (uMtCK)	Support of cardiac energy metabolism	Mediates the transfer of high energy phosphate from mitochondria to cytosol. Compensatory increase following oxidative stress has cardioprotective effects. ³⁵⁻³⁷
Connective tissue growth factor/ cysteine-rich 61/ nephroblastoma overexpressed family member 5 (CCN5)	Inhibition of cardiac fibrosis	Inhibits cardiac hypertrophy. Interferes with endothelial-mesenchymal transition and fibroblast-to-myofibroblast transdifferentiation, thereby reducing cardiac fibrosis. Prevents structural and electrical remodeling. Knockout leads to cardiomyopathy. ^{38,39}
Adipocyte fatty acid-binding protein 4 (AFABP4)	Suppression of cardiac contractility	Lipid binding protein that reduces fatty acid uptake by myocardium, thus suppresses cardiac contractility. ^{40,41} May act as indicator of increased sirtuin-1 signaling and autophagic flux. ⁴²
Retinoic acid receptor responder protein 2 (Rarres2, chemerin)	Promotion of cardiac hypertrophy, apoptosis and angiogenesis	Adipokine that induces cardiomyocyte hypertrophy, apoptosis and angiogenesis. ^{43,44} May act as biomarker of renal function, being inversely related to glomerular filtration rate. ⁴⁵
Proteins With Effects on the Kidneys		
Carbonic anhydrase 2 (CA2)	Renal tubular sodium, bicarbonate and water homeostasis	Binds to and enhances activity of NHE3 in proximal renal tubule. ⁴⁶ May offset increases in urinary bicarbonate and water resulting from SGLT2 inhibition.
Guanylin (GUCA)	Renal tubular sodium transport	After dietary salt load, guanylin and uroguanylin promote natriuresis by inhibiting NHE3 in proximal tubule. ^{47,48}
Uromodulin (UMOD)	Renal tubular sodium transport and muting of renal inflammation	Promotes sodium reabsorption in thick ascending limb of loop of Henle. ^{49,50} Acts as a trap for proinflammatory renal cytokines; loss-of-function mutations lead to chronic kidney disease. ⁵¹
Kidney injury molecule-1 (KIM-1)	Promotion of renal proximal tubular injury, inflammation and fibrosis	Mediates renal tubular cell injury and apoptosis and promotes tubulointerstitial inflammation and fibrosis. ⁵²⁻⁵⁴

Epithelial cell adhesion molecule (EpCAM)	Promotion of renal tubular integrity and regeneration	Promotes adhesion and polarity of renal tubular cells. EpCAM is suppressed by nephrotoxic agents and is enhanced during renal tubular regeneration ^{55,56}
Thymosin beta-10 (TMSB10)	Promotion of renal tubular integrity and regeneration	Plays important role in organization of cytoskeleton, thus promoting adhesion and polarity of renal parenchymal cells ^{57,58}
Beta-Ala-His dipeptidase (carnosinase 1, CNDP1)	Degradation of nephroprotective carnosine	Carnosine protects against the development of nephropathy, and gain-of-function mutations of carnosinase-1 cause carnosine depletion and increase risk of chronic kidney disease. ⁵⁹
Angiopoietin-like protein 2 (ANGPTL2)	Promotes renal inflammation and fibrosis	Expressed in endothelial cells. Promotes oxidative stress, inflammation and fibrosis in the kidney and heart. ^{60,61} May act as a marker of increased HIF-1 α activity. ⁶²

Proteins With Effects Other Than on the Heart and Kidneys

Promotilin (MLN)	No known action in heart or kidney	Prohormone for motilin, which promotes gastrointestinal motility. Produces relaxation of vascular smooth muscle, leading to systemic vasodilatation
Serine proteases inhibitor Kazal-type 1 (SPINK1)	No known action in heart or kidney	Inhibits trypsin with proposed role in the pathogenesis of pancreatitis. May be biomarker of renal function, since it increases in proportion to decreases in glomerular filtration rate
C-C motif chemokine 18 (CCL18)	No known action in heart or kidney	Expressed in lung, antigen-presenting dendritic cells and M2 macrophages and is chemoattractant for T cells and lymphocytes
C-C motif chemokine 27 (CCL 27)	No known action in heart or kidney	Expressed in skin and mediates homing of memory T lymphocytes to cutaneous sites
Cystatin F (CYTF, CST6)	No known action in heart or kidney	Inhibits cathepsin C-directed protease. Expressed in immune cells and causes down-regulation of killing efficiency of cytotoxic T lymphocytes
Scavenger receptor cysteine-rich domain-containing group B protein (SRCR)	No known action in heart or kidney	Expressed in epithelial cells and plays a role in mucosal immunity and innate defense

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RBP2 has effects on the heart and kidneys, but to avoid duplication, it is described only in the section on the heart.⁴ Abbreviations: AMPK= adenosine monophosphate-activated protein kinase; ATP= adenosine triphosphate; IGF1= insulin-like growth factor 1; NAD+= nicotinamide adenine dinucleotide; NHE3= sodium-hydrogen exchanger isoform 3; Wnt= Wingless-related integration site glycoprotein; HIF-1 α = hypoxia-inducible factor 1 α .

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