Positive effects of ferric iron on the systemic efficacy of nephrilin peptide in burn trauma

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Abstract

Introduction: Nephrilin peptide is a designed inhibitor of Rictor complex (also known as mTORC2), an evolutionarily conserved assembly believed to modulate responses to cellular stress. We previously demonstrated the ability of nephrilin peptide to suppress neuroinflammation, loss of body mass, glycaemic control and kidney function in a rat scald model, as well as sepsis mortality in a mouse model. The present study explores the effect of nephrilin plus iron formulations on clinically relevant outcomes in the rat scald model.

Methods: Animals were treated with nephrilin by subcutaneous bolus injection on post-burn days 1–7. Equimolar ferric iron in the formulation improved the positive systemic effects of nephrilin on kidney function, glycaemic control, oxidative stress, early hyperinflammation, late inflammasome activation, hyperangiogenesis and body mass, all variables previously shown to bear upon clinically relevant burn injury outcomes. The sparing effects of nephrilin-iron were demonstrated in both sexes.

Discussion: Surprisingly, optimum daily treatment doses were in the range of 2–4 mg/kg, while 8 mg/kg was less effective, suggesting the possibility of marginal pro-oxidant effects from the ‘free’ iron fraction. Thus, although ferric iron in the nephrilin formulation is clearly helpful, care must be exercised to select an optimum treatment dose.

Conclusion: Iron increases the efficacy of nephrilin peptide in burns.

Keywords
Nephrilin, trauma, burn injury, systemic, iron, hyperinflammation, kidney function, glycaemic control, pathological angiogenesis

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Introduction

Mammalian target of rapamycin (mTOR) is a serine threonine kinase that exists in two physically and functionally distinct assemblies, Raptor complex and Rictor complex (also known as mTORC1 and mTORC2). These complexes share some components such as TOR kinase but differ in terms of their regulation and cellular functions, as well as their responsiveness to the allosteric inhibitor, rapamycin.\(^1\) Rictor complex controls various members of the AGC sub-family of kinases, which includes Akt, serum and glucocorticoid-induced protein kinase 1 (SGK1), and protein kinases C-alpha and C-beta (PKC-\(\alpha/\beta\)). These kinases in turn regulate a range of cellular processes such as metabolism, survival, apoptosis, cytoskeletal architecture, cell motility, growth and proliferation by phosphorylating various effectors.\(^2,3\) The peptide nephrilin is a designed inhibitor of Rictor complex that is actively transported into cells in vivo.\(^4\) It binds to Rictor directly, preventing its binding to another component of the complex, Protor. Nephrilin was derived by fusing a 19 amino acid segment of Protor (a sequence common to the human, rat and mouse proteins) with the metal binding domain (MBD) of human insulin-like growth factor binding protein-3 (IGFBP3), a 21 amino acid sequence that targets and enters stressed cells.\(^5,6\)

One of the reported activities of Rictor complex is super-activation of Rac1 (Ras-related C3 botulinum toxin substrate 1), a small GTPase and obligate subunit of activated NADPH oxidase, which plays a central role in oxidative metabolism in skin, kidney and other tissues. Rac1 controls several cellular processes, including generation of oxidative radicals and reorganisation of the actin cytoskeleton in both normal cell types and cancer cells.\(^7\) The mechanism of action of nephrilin in rodent burn models appears to work through oxidative stress and Rac1 phosphorylation. Nephrilin treatment after burn injury reverses epigenetic and signalling changes in kidney tissue that lead to the activation of Rac1, and lowers elevations in markers of oxidative stress such as urinary 8-isoprostane and plasma OHDG. By inhibiting a stress-responsive function of Rictor complex, nephrilin blocks the gratuitous upregulation of Rac1 caused by severe stress without affecting basal levels of Rac1 activation.\(^8\) In theory, this represents a safer, more targeted intervention than, say, the use of TOR kinase inhibitors, whose effect upon housekeeping functions essential to cellular homeostasis are problematic.

Severe burn trauma is associated with a vast array of secondary effects including systemic inflammation, loss of lean body mass, sepsis, organ failure, loss of glycaemic control, delayed wound healing and cognitive deficits. These serious and enduring complications can lead to substantial morbidity and mortality.\(^9-13\) We previously showed the pleotropic effects of nephrilin peptide in combating post-burn systemic neuroinflammation, loss of glycaemic control, lean body mass and kidney function, and impaired wound healing in a rat scald model; and sepsis in a mouse model. Some, but not all, of these systemic effects of nephrilin appear to be influenced by the animal’s iron status.\(^14,15\) Severe trauma is associated with anaemia mediated by hepcidin, an iron-regulatory protein in serum and a current target of therapy in critical illness.\(^16\)

In the present study, we use a well-established rat scald model\(^17\) to explore the impact of a seven-day treatment regimen comprising subcutaneous bolus injection of nephrilin peptide with or without iron. The treatment begins after scald, in parallel with real-life injury. Nephrilin has previously been shown to modulate the neuroimmune response to a variety of xenobiotic and metabolic stressors in rodents.\(^18,19\) When injected into mice at high doses daily for 26 days, nephrilin generates no visibly differential pathology compared to vehicle.\(^18\) Nephrilin contains a metal-binding domain known to bind ferrous (Fe\(^{2+}\)) and ferric (Fe\(^{3+}\)) iron.\(^8\) Based on cross-linking studies, uptake of this metal-binding
domain into mammalian cells involves binding
to integrin-beta-3, a component of a major metal
uptake pathway, and to transferrin receptor.19,20

**Material and methods**

**Reagents**

Nephrilin peptide, a 40-mer peptide carrying a
sequence derived from PRR5/Protor (the
sequence is conserved in human, rat and mouse
species) was synthesised by Lifetein LLC
(Hillsborough, NJ, USA) and purified to > 80%
by high-performance liquid chromatography.
The design and synthesis of nephrilin have been
previously described. 4 Antibodies for ELISAs
were purchased from Abcam (Cambridge, MA,
USA) and chemicals from Sigma-Aldrich (St.
Louis, MO, USA) unless otherwise specified.

**Nephrilin administration**

Adult Sprague Dawley rats of both sexes (250–300
g, Charles River Laboratories, Wilmington, MA,
USA) were injected with nephrilin peptide plus
or minus equimolar metal (zinc, ferrous iron or
ferric iron) once daily by subcutaneous bolus
injection, days 1–7. Treatment group sizes were n
= 8 for each sex unless otherwise indicated:
group S = sham-treated; group B = burn + vehi-
cle; group N1 = burn + 4 mg/kg nephrilin;
group N1/Zn2 = burn + 4 mg/kg nephrilin/zinc
chloride; group N1/Fe2 = burn + 4 mg/kg
nephrilin/ferrous sulphate; and groups N1/Fe3
(2, 4 or 8) = burn + 2, 4, or 8 mg/kg nephrilin/equimolar ferric chloride. The first dose
was administered after completion of the scald pro-
cedure. Injection volume was 400 μL. Control an-
imals received the same volume of vehicle. The 4
mg/kg daily dosage of nephrilin was selected
based on its demonstrated safety and efficacy in
11 different rodent disease models tested to date
(unpublished data).7–10,11,14 In a non-GLP study,
mice that were treated daily with 20 mg/kg
nephrilin by subcutaneous bolus for 26 days
showed no differential toxicity in major organs
when compared to a saline control.4

**Rat scald model**

The rat scald burn model17 is a modified Walker-
Mason model that induces inflammation and
hypermetabolism in line with what severely
burned patients experience. The model results in
a mortality rate of < 1%. Adult male Sprague
Dawley rats were housed in clean cages on a 12-h
light/dark cycle with access to food (standard
chow) and water ad libitum. Animals were allowed
to acclimate for one week before the experiment.
All animal procedures were performed in adher-
ence to the National Institute of Health’s Guide for
Care and Use of Laboratory Animals and approved by
the Institutional Animal Care and Use Committee
(IACUC) of the Molecular Medicine Research
Institute. All procedures were initiated in the
morning between 07:00 h and 10:00 h. Prophylactic analgesia (0.05 mg/kg body weight
Buprenorphin) was administered 15 min before
general anaesthesia using isofluorane. The dor-
sum of the trunk and the abdomen were shaved,
and a 60% of total body surface area (TBSA) burn
administered by placing the animals in a mould
and immersing them in water heated to 98–100
°C for 10 s on the back and 2 s on the abdomen,
except that for anatomical reasons female rats
received only the dorsal burn, thereby reducing
the burn exposure for female rats. This method
delivers a full-thickness cutaneous burn as con-
firmed by histological examination. Burned rats
were immediately resuscitated with 40 cc/kg
Ringer’s Lactate injected intraperitoneally. Animals in the sham group were treated exactly
as described above for burned animals except
that the animals were placed in room tempera-
ture water. Animals were randomly assigned to
treatment groups, and nephrilin (4 mg/kg) or
saline were administered by subcutaneous bolus
daily. Each treatment group comprised 16 ani-
mals (eight of each sex). At the end of the study
period, animals were euthanised by decapitation
as approved by MMRI IACUC guidelines, the
NIH’s Office of Laboratory Animal Welfare
(OLAW) and AVMA recommendations. All tis-
tues and organs of interest were rapidly dissected
or collected and flash frozen in liquid nitrogen
with subsequent storage at –80 °C.

**Glucose tolerance test**

Fourteen days post-burn, the rat tail was snipped
and the baseline glucose level measured using a
BAYER contour blood glucose monitoring sys-
tem. The rats were injected intraperitoneally with
glucose and readings were performed at 30 min,
60 min and 120 min after injection. Results were
expressed as areas under the curve (μg/dL/h)
over baseline over the sampling period.

**Early hyperinflammation and
inflammasome activation**

Twenty-four hours after scald, a blood sample was
taken from each (isofluorane anaesthetised) rat.
Plasma IL-6 was measured using a rat IL-6 DuoSet
ELISA kit (R&D Systems, Minneapolis, MN, USA). Blood taken at 14 days post-burn was analysed for 27 cytokine and chemokine analytes including VEGF-A, IL-18, IL1-beta, CCL5 and CXCL5 (RD27 Custom Plex Discovery Assay, Eve Technologies, Calgary, AB, Canada).

**Plasma OHDG**

Fourteen-day plasma was assayed for OHDG using an Oxidative Damage High Sensitivity ELISA Kit purchased from Cayman Chemical (Ann Arbor, MI, USA).

**Kidney function (plasma creatinine)**

Kidney function was indirectly assessed by measuring 14-day plasma creatinine. Estimated glomerular filtration rate (eGFR; mL/min/100 g animal body weight) can be computed from this value as previously described.15,21

**PctRedPix computation**

A digital image of each wound at two weeks post-scaled were analysed using GIMP 2.10 software. Red pixels, as a percentage of all pixels within the wound area, were counted by the software and expressed as a percentage of total.

**Computation of efficacy**

Aggregate efficacy of each treatment regimen was computed using an average of Z-scores calculated from the distribution of values for each analyte by subtracting the mean of the distribution from the value and dividing by the standard deviation for the distribution.

**Statistical analysis**

Data are presented as means ± SD unless otherwise indicated. P values were computed using Student’s t-test and expressed relative to sham or saline-treated group.

**Results**

Traditionally, in this model, male rats have been used. To our knowledge, the absence of published data on female rats reflects the possible confounding effects of oestrous cycles, as well as anatomical differences necessitating changes in scald procedure (see ‘Materials and methods’). We therefore tested treatment regimens on male rats first and confirmed the efficacy of the most informative treatment regimens on female rats. We did not observe effects in two of the seven classes (hyper-angiogenesis, weight loss), possibly because the modified scald for female rats was milder. Thus, aggregate efficacy computations for male rats averaged seven efficacy classes and for female rats, five classes. A comparison aggregate efficacy plot for both sexes in shown in Figure 1.

**Male rats: ferric iron improves efficacy of nephrilin peptide**

Table 1 shows the results obtained when male rats in the scald model were exposed to vehicle, 4 mg/kg nephrilin and 4 mg/kg nephrilin complexed with either ferric iron, ferrous iron or zinc. To make global comparisons of treatment efficacy, we converted values to z-scores. All scores within an efficacy class were averaged, allowing allocation of equal weight to efficacy classes. A composite z-score (average z score across all seven effect classes) is shown in the last row of the table. This allows direct comparison of efficacy across treatment groups. The sham treatment involves manipulating the animals exactly as in the burn group, but without the burn treatment. The efficacy value for the sham (0.82) is thus the positive control. The negative control is the Burn + vehicle group (−0.61). The results show that ferric iron supplementation is superior to the other metals (efficacy of 0.27 vs. 0.01 and −0.19 for zinc and ferrous iron, respectively) in improving the efficacy of nephrilin on the following clinically relevant readouts: early hyperinflammation (24-h
plasma IL-6), inflammasome activation at two
weeks (IL-18, IL-1-beta), hyperangiogenesis
(VEGF-A, CCL5, CXCL5, PctRedPix), glycaemic
control, kidney function, oxidative stress and
weight loss. Compared to vehicle or nephrilin
treatment alone (−0.31), nephrilin with ferric
iron (0.27) is at least twice as efficacious overall.

Male rats: dose ranging of nephrilin-ferric
iron treatment

Table 1 shows the results obtained when male
rats in the scald model were exposed to vehicle,
2, 4 and 8 mg/kg nephrilin complexed with fer-
ric iron. The results show that 2 mg/kg (0.17)
and 4 mg/kg (0.27) dose levels were superior to
8 mg/kg (−0.37). This surprising result may indi-
cate that, at high doses, the pool of ‘free’ iron in
equilibrium may reach detrimental levels.

Female rats: nephrilin in female rats
shows improved efficacy with ferric iron

Table 2 shows the results obtained when female
rats in the scald model were exposed to vehicle
or nephrilin complexed with ferric iron. As in
male rats, ferric iron increased the potency of 4
mg/kg nephrilin (2.45 vs. 0.48) in the presence
of ferric iron. This result confirms the effect of
ferric iron seen in male animals.

A similar pattern of improvement is seen
with ferric iron supplementation in both
sexes

Figure 1 shows a composite of the efficacies com-
puted for key treatments in both sexes. A similar
pattern of efficacy is seen for ferric iron supple-
mentation in the dose range of 2–4 mg/kg for
both sexes.

Discussion

In rodent models of stress, nephrilin peptide has
been shown to reverse elevations in neuroim-
mune and oxidative stress consequent to ther-
mal, metabolic and xenobiotic insult. Dysregulated host immune responses to traum-
atic stress, massive infection and other severe challenges sometimes exhibit a time-course of hyperinflammation—often leading to multiple organ failure, sepsis or death—followed by chronic critical illness, recently dubbed Persistent Inflammation, Immunosuppression and Catabolism Syndrome (PICS). Each of our ear-
lier findings regarding the robust effects of nephri-
lin peptide on early IL-6 elevation, and other

represents an alarming and rapidly expanding
burden on the healthcare system. Nephrilin’s
efficacy in reversing the systemic effects of sepsis
and burn trauma, including loss of glycaemic
control, body mass, kidney function, wound
healing capacity and sepsis appears to involve this pleiotropic dysfunction.

In recent years, our understanding of PICS
has evolved. Readouts such as elevated plasma
IL-6 at 24 h post-insult, chronic inflammasome
activation, loss of body mass, wound healing
impaired by excessive angiogenesis, loss of glycae-
mic control, elevated markers of oxidative stress,
immune dysregulation and neurodegenerative
consequences, among others, have emerged as
emblematic of the progression of PICS.

In a previous study, the possible effect of
iron in the efficacy of nephrilin peptide in a rat
scald model was suggested. Here we show that, in
both sexes of rats, the addition of equimolar fer-
ric iron (but not ferrous iron or zinc) roughly
doubles the efficacy of the peptide in the model.
However, the optimal dose range for the dosing
regimen adopted (subcutaneous bolus injection,
once daily, for seven days after burn) appears to
be 2–4 mg/kg/day. At a higher dose (8 mg/kg/ 
day), a reduced efficacy was observed. One pos-
sible explanation for the lowered efficacy
observed at high doses of nephrilin-ferric-iron
(N1/Fe3) formulations is that the ‘free’ pool of
iron expected to equilibrate into circulation has
some negative effect, such as increasing oxidative
stress. In support of this hypothesis, elevated
plasma OHDG levels were not reduced at the
higher dose.

NLRP3 inflammasome activation in burn
injury appears to be a double-edged sword: a
small amount may be helpful to healing, but
excessive activation is associated with poor out-
comes. We showed that N1/Fe3 treatments sig-
ificantly reduced plasma levels of IL-18 and
IL-1-beta, markers of NLRP3 inflammasome
activation. Similarly, although angiogenesis is benefi-
tial to early stages of wound healing, pathological
angiogenesis may reduce capacity for complete
wound healing. Our readouts of pathological
angiogenesis included VEGF-A, CCL5, CXCL5
and a visual measure of percent red pixels in
wounds, PctRedPix. N1/Fe3 treatments at 2–4
mg/kg significantly reduced readouts in this
class. To our knowledge, this is the first demon-
stration of the use of this class of readouts in a
burn model.

The present study confirmed each of our ear-
ier findings regarding the robust effects of nephri-
lin peptide on early IL-6 elevation, and other
Table 1. Efficacy in male rats.

| Effect class | S (Sham) | B (vehicle) | N1 (4 mg/kg) | N1/Zn2 (4 mg/kg) | N1/Fe2 (4 mg/kg) | N1/Fe3 (2 mg/kg) | N1/Fe3 (4 mg/kg) | N1/Fe3 (8 mg/kg) |
|--------------|----------|-------------|--------------|------------------|------------------|------------------|------------------|------------------|
| 1            | 31.4 ± 12.0" | 62.3 ± 16.9 | 50.6 ± 6.1   | 43.4 ± 13.6"     | 49.1 ± 13.1     | 38.5 ± 27.8      | 41.4 ± 11.2"     | 48.5 ± 18.5      |
| 2a           | 125.2 ± 45.5" | 203.8 ± 61.8 | 178.1 ± 19.3 | 133.3 ± 25.1     | 157.7 ± 70.7    | 220.4 ± 32.1     | 119.1 ± 15.4"    | 148.2 ± 61.7      |
| 2b           | 41.9 ± 12.3"  | 266.8 ± 185.2| 124.3 ± 132  | 36.7 ± 9.5"      | 97.4 ± 86.6     | 52.9 ± 7.8"      | 36.6 ± 7.8"      | 254.5 ± 291.9     |
| 3a           | 50.4 ± 10.2"  | 67.4 ± 17.4  | 56.1 ± 11.7  | 38.3 ± 8.8"      | 41.2 ± 6.8"     | 37.8 ± 15.3"     | 48.1 ± 10.9"     | 43.3 ± 8.1"       |
| 3b           | 218.1 ± 41.8" | 664.8 ± 42.8 | 609.7 ± 283  | 321.1 ± 96"      | 249.8 ± 73"     | 248.3 ± 29.7"    | 192.1 ± 26.3""   | 301.2 ± 74.9      |
| 3c           | 763 ± 229"    | 2009 ± 745   | 1910 ± 380   | 1154 ± 224"      | 939 ± 300""     | 855 ± 233"""     | 1038 ± 495""     | 1435 ± 445"       |
| 3d           | n/a         | 20.01 ± 1.37 | 16.51 ± 3.02 | 9.74 ± 5.79"     | 11.79 ± 5.6""   | 6.53 ± 3.16""    | 8.65 ± 3.35"""   | 18.3 ± 11.83"     |
| 4            | 78.4 ± 43.4" | 157.3 ± 69.8 | 139.3 ± 48.6 | 215.6 ± 124"     | 120.6 ± 65.4    | 136.4 ± 69.2     | 94.3 ± 25.4""    | 93.8 ± 37.2""     |
| 5            | 0.23 ± 0.03" | 0.38 ± 0.18  | 0.34 ± 0.13  | 0.26 ± 0.12"     | 0.38 ± 0.16"    | 0.31 ± 0.09"     | 0.18 ± 0.11"""   | 0.38 ± 0.12""     |
| 6            | 479 ± 116"   | 665 ± 78     | 585 ± 137    | 454 ± 76"""       | 455 ± 71"""      | 374 ± 187""""     | 423 ± 79""""      | 761 ± 89""""       |
| 7            | 5.58 ± 1.00" | 2.34 ± 1.03  | 2.32 ± 1.01  | 1.66 ± 1.08"     | 2.65 ± 0.56"    | 2.99 ± 0.53"     | 2.71 ± 1.62"     | 2.50 ± 0.55""     |
| Average      | 0.82 ± 0.58" | −0.61 ± 0.47 | −0.31 ± 0.35 | 0.01 ± 0.62""    | −0.19 ± 0.52""  | 0.17 ± 0.40"""    | 0.27 ± 0.60"""    | −0.37 ± 0.45""    |

Treatment groups (n=8) are described in the 'Materials and methods' section. Effect classes: 1 = early inflammation (24-h plasma IL-6 pg/mL); 2 = 14-day plasma inflammasome markers (2a = IL18 pg/mL; 2b = IL1b pg/mL); 3 = 14-day plasma hyperangiogenesis markers (3a = VEGF-A pg/mL; 3b = CCL5 pg/mL; 3c = CXCL5 pg/mL; 3d = PctRedPix); 4 = glycaemic control, 14-day GTT (AUC mg.dL.h); 5 = kidney function, 14-day plasma (creatinine mg/dL); 6 = systemic oxidative stress, 14-day plasma (OHDG pg/mL); 7 = weight loss (slope). *P < 0.05 vs. B group. †P < 0.05 vs. N1 group. ‡P < 0.05 vs. 8 mg/kg group.
clinically relevant effects on glycaemic control, kidney function, loss of body mass and systemic oxidative stress in the rat scald model. The present study further showed efficacy of nephrilin for both sexes in most of these effect classes. This is the first time that the efficacy of nephrilin in this model has been tested in female rats. Perhaps because of anatomical differences (which affect scald exposure) and possible confounding influences of the oestrous cycle, female rats have rarely been investigated in this type of rat scald model. Our results suggest that the improved efficacy of treatments with nephrilin-iron versus nephrilin alone were similar in both sexes.

Our results also raise obvious questions for future study. Is it possible to modify the iron-binding properties of this peptide so as to allow for tighter binding of the metal and possibly higher dose efficacy? Can this peptide be coupled to other moieties to improve effective treatment of burn trauma? We intend to address these questions in future experiments.

### Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This project was supported by the following grants: 1R43GM151424 to DDM.

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### Table 2. Efficacy in female rats.

| Effect class | S (Sham) | B (vehicle) | N1 (4 mg/kg) | N1/Fe3 (2 mg/kg) | N1/Fe3 (4 mg/kg) |
|-------------|----------|------------|-------------|----------------|----------------|
| 1           | 46 ± 13  | 63 ± 15    | 51 ± 9      | 51 ± 12        | 36 ± 15*        |
| 2           | 129.6 ± 24.1* | 170.4 ± 28.8 | 154.1 ± 30.3 | 155.2 ± 39.9 | 137.0 ± 19.4*   |
| 4           | 55.9 ± 17.9* | 95.1 ± 40.3 | 67.5 ± 19.8 | 79.2 ± 21.7 | 52.3 ± 19.2*    |
| 5           | 0.26 ± 0.02* | 0.31 ± 0.03 | 0.24 ± 0.04* | 0.22 ± 0.07* | 0.26 ± 0.03*    |
| 6           | 216 ± 116* | 765 ± 152  | 633 ± 263   | 363 ± 251* | 452 ± 147*      |
| Average     | 2.17 ± 1.27* | −1.52 ± 2.67 | 0.48 ± 0.96 | 1.10 ± 1.31 | 2.45 ± 2.04*    |

Treatment groups (n=8) are described in the ‘Materials and methods’ section.

Effect classes: 1 = early inflammation (24h plasma IL-6 pg/mL); 2 = 14-day plasma inflammasome marker IL18 pg/mL; 4 = glycaemic control, 14-day GTT (AUC mg.dL.h); 5 = kidney function, 14-day plasma (creatinine mg/dL); 6 = systemic oxidative stress, 14-day plasma (OHDG pg/mL).

*P < 0.05 vs. B group.
†P < 0.05 vs. N1 group.
8

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How to cite this article
Mascarenhas DD, El Ayadi A, Ravikumar P, Kang GJ, Langer T, Moreno C and Amento EP. Positive effects of ferric iron on the systemic efficacy of nephrilin peptide in burn trauma. *Scars, Burns & Healing*, Volume 6, 2020. DOI: 10.1177/2059513118928494.