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Nowe biomarkery zaburzeń mikrokrążenia w twardzinie układowej.

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne

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Key words: systemic sclerosis, biomarker, copeptin, Hypoxia-Inducible Factor 1α (HIF1α), microangiopathy, Raynaud's phenomenon, scleroderma renal crisis (SRC)

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WYKAZ PUBLIKACJI STANOWIĄCYCH PRACĘ DOKTORSKĄ

Lp.	Artykuł	IF	Punkty MNiSW
1	**Chrabaszcz M.**, Małyszko J., Sikora M., Alda-Malicka R., Stochmal A., Matuszkiewicz-Rowińska J., Rudnicka L. Renal Involvement in Systemic Sclerosis: An Update. *Kidney Blood Press Res.* 2020;45(4):532-548.	2,687	70
2	**Maciejewska M.**, Sikora M., Maciejewski C., Alda-Malicka R., Czuwara, J., Rudnicka L., Raynaud's phenomenon with focus on systemic sclerosis. *J. Clin. Med.* 2022, 11, 2490.	4,964	140
3	**Maciejewska M**., Stec A., Zaremba M., Maciejewski C., Rudnicka L., Sikora M Copeptin as a biomarker of microcirculation alterations in systemic sclerosis *Clin Cosmet Investig Dermatol.* 2023;16:1351-1361	2.765	100
4	**Maciejewska M.**, Sikora M., Stec A., Pawlik K., Zaremba M., Rudnicka L. Hypoxia-inducible factor-1α (HIF-1α) as a biomarker for changes in microcirculation in individuals with systemic sclerosis. *Dermatol Ther (Heidelb) 2023* https://doi.org/10.1007/s13555-023-00952-w	3,661	100
	Łącznie	**14,077**	**410**

SPIS TREŚCI

1. WYKAZ STOSOWANYCH SKRÓTÓW

ACA *(ang. anti-centromere antibodies)* - przeciwciała antycentromerowe (przeciw białkom kinetochorowym)
ACR *(ang. American College of Rheumatology)* - Amerykańskie Towarzystwo Reumatologiczne
ANA *(ang. antinuclear antibodies)* - przeciwciała przeciwjądrowe
anty-PM/Scl *(ang. anti-PM/Scl, anti-Polymyositis-Scleroderma antibodies)* przeciwciała przeciw PM/Scl (kompleksowi białek jąderkowych o aktywności rybonukleazy)
anty-RNAP III *(ang. anti-RNA polymerase III antibodies)* - przeciwciała przeciw polimerazie III RNA
anty-TOPO I *(ang. anti-topoizomerase I antibodies)* - przeciwciała przeciw topoizomerazie I
dcSSc *(ang. diffuse cutaneous systemic sclerosis)* - twardzina układowa z uogólnionymi stwardnieniami skóry
DLCO *(ang. diffusing capacity of the lung for carbon monoxide)* - dyfuzja tlenku węgla w płucach
DUs *(ang. digital ulcers)* - owrzodzenia paliczków rąk
ELISA *(ang. enzyme-linked immunosorbent assay)* - test immunoenzymatyczny
EULAR *(ang. European League Against Rheumatism)* - Europejska Liga do Walki z Reumatyzmem
GFR *(ang. glomerular filtration rate)* - przesączanie kłębuszkowe
HIF-1 *(ang. Hipoxia induced factor)* - czynnik indukowany hipoksją-1
HRCT *(ang. high resolution computed tomography)* tomografia komputerowa wysokiej rozdzielczości
ILD *(interstitial lung disease)* - śródmiąższowa choroba płuc
lcSSc *(ang. limited cutaneous systemic sclerosis)* - twardzina układowa z ograniczonymi stwardnieniami skóry
mRSS - *(ang. modified Rodnan skin thickness score)* **-** zmodyfikowana skala Rodnana
NVC – *(ang. nailfold videocapillaroscopy)* - kapilaroskopia wału paznokciowego
PAH *(ang. pulmonary arterial hypertension)* - tętnicze nadciśnienie płucne
PGI2 *(ang. prostaglandin I2)* - prostaglandyna I2
RCS *(ang. Raynaud's Condition Score)* - skala nasilenie objawu Raynauda

SSc (ang. *Systemic Sclerosis* – SSc) - twardzina układowa

TNF-α *(ang. tumor necrosis factor-α)* - czynnik martwicy nowotworów alfa

TGF-β *(ang. transforming growth factor-β)* - transformujący czynnik wzrostu beta

VEGF *(ang. vascular endothelial growth factor*) - naczyniowo-śródbłonkowy czynnik wzrostu

2. STRESZCZENIE

NOWE BIOMARKERY ZABURZEŃ MIKROKRĄŻENIA W TWARDZINIE UKŁADOWEJ

2.1. Wprowadzenie

Twardzina układowa jest autoimmunologiczną chorobą tkanki łącznej związaną z postępującym włóknieniem skóry i narządów wewnętrznych. Zgodnie z aktualną koncepcją patogenezy choroby, początkowo dochodzi do uszkodzenia komórek śródbłonka, zaburzeń mikrokrążenia i rozwoju stanu zapalnego. Sam proces włóknienia wydaje się wtórnym do stymulacji przez cytokiny uwalniane ze śródbłonka. Kliniczną manifestacją zaburzeń krążenia są m.in.: przełom nerkowy, nadciśnienie płucne lub objaw Raynauda, który niejednokrotnie wyprzedza pojawienie się innych cech twardziny układowej nawet o kilka lat, stwierdzany u ponad 95% chorych. Krytyczne niedokrwienie wraz z owrzodzeniami dystalnych części palców stanowi najcięższą postać zaburzeń mikrokrążenia, prowadząc do wystąpienia bólu, niepełnosprawności oraz zmniejszenia jakości życia.

Wczesne rozpoznanie i leczenie nieprawidłowości naczyniowych w twardzinie układowej ma zasadnicze znaczenie dla poprawy wyników leczenia pacjentów i zapobiegania długotrwałym powikłaniom. W ostatnich latach trwają poszukiwania biomarkerów szybkiej progresji zmian naczyniowych, które odpowiednio wcześnie pozwoliłyby na intensyfikację leczenia reologicznego.

2.2. Cele pracy

Celem pracy była analiza zaburzeń mikrokrążenia prowadzącego do objawu Raynauda i patologii nerek u pacjentów z twardziną układową oraz poszukiwanie nowych biomarkerów związanych z uszkodzeniem naczyń w przebiegu twardziny układowej, w tym w szczególności:

1) Ocena możliwości wykorzystania kopeptyny i czynnika indukowanego hipoksją-1 α (HIF-1α) jako potencjalnych biomarkerów zaburzeń mikrokrążenia w twardzinie układowej
2) Ocena potencjalnej korelacji między stężeniem kopeptyny oraz czynnika indukowanego hipoksją-1α i aktywnością twardziny układowej
3) Analiza wpływu leczenia reologicznego alprostadylem na stężenie wybranych biomarkerów zaburzeń mikrokrążenia u pacjentów z twardziną układową

2.3. Materiał i metody

Do badań przedstawionych w cyklu publikacji włączono 50 pacjentów z rozpoznaną twardziną układową spełniających kryteria klasyfikacyjne ACR/EULAR. Do grupy kontrolnej włączono 30 zdrowych ochotników dobranych pod względem wieku i płci.

Zajęcie narządowe oceniano według rekomendacji diagnostyczno-terapeutycznych Polskiego Towarzystwa Dermatologicznego. Zaburzenia naczyniowe oceniano na skórze gładkiej twarzy i kończyn (liczba i lokalizacja owrzodzeń paliczków, teleangiektazji), oraz wykonanie badania kapilaroskopowego z oceną zaburzeń mikrokrążenia wg klasyfikacji Cutolo.

Jako potencjalne biomarkery zaburzonego mikrokrążenia obwodowego wybrano: czynnik indukowany hipoksją-1α (HIF-1α) oraz kopeptynę. Stężenia HIF-1α i kopeptyny oceniono przy pomocy metody ELISA. Poziom istotności statystycznej przyjęto dla $p<0,05$.

2.4. Wyniki

Pacjenci z twardziną układową mieli znacznie wyższe stężenie kopeptyny w osoczu (4,21 pmol/L [3,04-5,42]) w porównaniu do grupy kontrolnej (3,40 pmol/L [2,38-3,76], $p<0,01$). Stwierdzono dodatnią korelację pomiędzy nasileniem objawu Raynauda a stężeniem kopeptyny ($r=0,801$, $p<0,05$). Pacjenci „późnym" wzorcem zmian naczyniowych w kapilaroskopii (5,37 pmol/l [4,29-8,06]) charakteryzowali się wyższymi wartościami kopeptyny niż pacjenci o „wczesnym" (2,43 pmol/l [2,25-3,20], $p<0,05$) i aktywnym wzorcu (3,93 pmol/l [2,92-5,16], $p<0,05$). Pacjenci z twardzina układową, u których występowały owrzodzenia paliczków palców mieli istotnie statystycznie wyższe stężenie kopeptyny (5,71 pmol/l [IQR 4,85-8,06]) niż pacjenci bez owrzodzeń na paliczkach palców (3,31 pmol/l, [2,28-4,30], $p<0,05$). Ponadto stężenie kopeptyny charakteryzowało się dobrą dokładnością diagnostyczną w rozróżnianiu pacjentów z owrzodzeniem palców i bez (AUC=0,863). Dożylne leczenie analogami prostaglandyn (alprostadylem) powodowało zmniejszenie stężenia kopeptyny z 4,96 [4,02-6,01] do 3,86 pmol/l [3,17-4,63] ($p<0,01$) po 4-6 cyklach podawania.

Pacjenci z twardziną układową mieli istotnie statystycznie wyższe stężenie czynnika indukowanego hipoksją-1α (3,042 ng/ml [2,295-7,749]) w porównaniu z grupą kontrolną (1,969 ng/ml [1,531-2,903] $p<0,01$). Pacjenci z uogólnioną postacią twardziny układowej (2,803 ng/ml, IQR 2,221-8,799) i formą ograniczoną (3,231 ng/ml, IQR 2,566-5,502) wykazywali zwiększone stężenie czynnika indukowanego hipoksją-1α w porównaniu z grupą kontrolną ($p<0,01$). Stwierdzono statystycznie wyższe stężenie czynnika indukowanego

hipoksją-1α u pacjentów z "aktywnym" kapilaroskopowym obrazem twardzinowym wg klasyfikacji Cutolo (6,625 ng/ml, IQR 2,488-11,480) w porównaniu z pacjentami z obrazem „wczesnym” (2,739, IQR 2,165-3,282, $p<0,05$) i „późnym” (2,983 ng/ml, IQR 2,229-3,386, $p<0,05$). Jednocześnie stwierdzono, że pacjenci, u których nie występowały owrzodzenia paliczków palców, mieli znacząco wyższe stężenie czynnika indukowanego hipoksją-1α (4,367 ng/ml, IQR 2,488-9,462) niż pacjenci z aktywnymi owrzodzeniami paliczków palców (2,832 ng/ml, IQR 2,630-3,094, $p< 0,05$) oraz owrzodzeniami paliczków palców w przeszłości (2,668 ng/ml, IQR - 2,074-2,983, $p<0,05$).

2.5. Wnioski

1. Kopeptyna i czynnik indukowany hipoksją-1α (HIF-1α) mogą posłużyć jako potencjalne biomarkery zaburzeń mikrokrążenia obwodowego u pacjentów z twardziną układową
2. Stężenie kopeptyny w surowicy wykazuje pozytywną korelację z nasileniem objawu Raynauda i występowaniem owrzodzeń obwodowych w przebiegu twardziny układowej oraz jest czułym parametrem odpowiedzi na leczenie reologiczne
3. Stwierdzono statystycznie wyższe stężenie czynnika indukowanego hipoksją-1α w surowicy u pacjentów z "aktywnym" kapilaroskopowym obrazem twardzinowym wg klasyfikacji Cutolo w porównaniu z pacjentami z obrazem „wczesnym” i „późnym”
4. Monitorowanie stężenia kopeptyny i czynnika indukowanego hipoksją-1α u pacjentów z twardziną układową może pozwolić na identyfikację pacjentów o wysokim ryzyku nasilonych zaburzeń mikrokrążenia i tym samym wczesnego rozpoczęcia intensywnego leczenia reologicznego

3. SUMMARY

NEW BIOMARKERS OF MICROVASCULAR IMPAIRMENT IN SYSTEMIC SCLEROSIS.

3.1. Introduction

Systemic sclerosis is an autoimmune connective tissue disease associated with progressive fibrosis of the skin and internal organs. According to the current concept of disease pathogenesis, endothelial cell damage, microcirculation disorders, and inflammation develop initially. The process of fibrosis itself seems to be secondary to stimulation by cytokines released from the endothelium. Clinical manifestations of circulatory disorders include renal crisis, pulmonary hypertension, or Raynaud's phenomenon, which often precedes the appearance of other features of systemic sclerosis by several years and is found in over 95% of patients. Critical ischemia together with ulceration of the distal parts of the fingers is the most severe form of microcirculation disorder, leading to pain, disability, and reduced quality of life.

Currently, it is challenging to identify patients at risk of rapid disease progression and development of severe organ complications at an early stage. For these reasons, the aim of the study is to search for new biomarkers associated with vascular damage in the course of systemic sclerosis, to assess their relationship with disease activity and response to vascular therapy.

3.2. Objective

The aim of the study was to analyze microcirculatory disorders leading to Raynaud's phenomenon and renal pathology in patients with systemic sclerosis, as well as to search for new biomarkers associated with vascular damage in systemic sclerosis, including in particular:

1. Evaluation of the potential use of copeptin and hypoxia-inducible factor-1α (HIF-1α) as biomarkers for microcirculatory disorders in systemic sclerosis.
2. Assessment of the potential correlation between the concentration of copeptin and hypoxia-inducible factor-1α and the activity of systemic sclerosis.
3. Analysis of the impact of rheological treatment with alprostadil on the concentration of selected biomarkers of microcirculatory disorders in patients with systemic sclerosis.

3.3. Material and methods

50 patients with systemic sclerosis and qualified for rheological treatment with alprostadil have been enrolled in the study according ACR/EULAR classification criteria. The control group included 30 healthy volunteers who were matched for age and sex. Organ involvement was assessed according to the diagnostic and therapeutic recommendations of the Polish Society of Dermatology. Vascular disorders have been assessed on skin of the face and limbs (number and location of digital ulcers, telangiectasia), nailfold capillaroscopy image classified according to Cutolo scale.

Hypoxia-inducible factor-1α (HIF-1α) and copeptin were selected as potential biomarkers for analysis. HIF-1 and copeptin concentrations were assessed by ELISA. Statistical significance level was assumed for $p<0.05$.

3.4. Results

We found significantly higher copeptin concentration in patients with systemic sclerosis (4.21 pmol/L [3.04-5.42]) in comparison to control group (3.40 pmol/L [2.38-3.76], $p<0.01$). Copeptin significantly correlated with Raynaud's condition score ($r=0.801$, $p<0.05$). Patients with "late" capillaroscopic patterns had higher copeptin concentrations (5.37 pmol/L [4.29-8.06]) than patients with "early" (2.43 pmol/L [2.25-3.20], $p<0.05$) and "active" patterns (3.93 pmol/L [2.92-5.16], $p<0.05$]). Copeptin was found to be significantly higher in systemic sclerosis patients with DUs (5.71 pmol/L [IQR 4.85–8.06]) when compared to systemic sclerosis patients without DUs (3.31 pmol/L, [2.28-4.30], $p<0.05$). Additionally, copeptin concentration had good diagnostic accuracy in discriminating between patients with and without digital ulcers (AUC=0.863). Alprostadil decreased copeptin concentration from 4.96 [4.02-6.01] to 3.86 pmol/L [3.17-4.63] ($p<0.01$) after 4-6 cycles of administration.

The results show a marked increase in hypoxia-inducible factor-1α levels in patients with systemic sclerosis (3.042 ng/ml [2.295-7.749]) when compared to the control group (1.969 ng/ml [1.531-2.903] $p<0.01$). Patients with both, diffuse cutaneous SSc (2.803 ng/ml, IQR 2.221-8.799) and limited cutaneous SSc (3.231 ng/ml, IQR 2.566-5.502) displayed an increased serum hypoxia-inducible factor-1α levels compared to the control group ($p<0.01$). We found a notable increase in hypoxia-inducible factor-1α plasma concentration in patients with an "active" pattern (6.625 ng/ml, IQR 2.488-11.480) compared to those with either an "early" pattern (2.739, IQR 2.165-3.282, $p< 0.05$) or a "late" pattern (2.983 ng/ml, IQR 2.229-3.386, $p<0.05$). Patients with no history of digital ulcers had significantly higher levels of hypoxia-

inducible factor-1α (4.367 ng/ml, IQR 2.488-9.462) compared to patients with either active digital ulcers (2.832 ng/ml, IQR 2.630-3.094, $p<0.05$) or healed digital ulcers (2.668 ng/ml, IQR - 2.074-2.983, $p<0.05$).

3.5. Conclusion

1. Copeptin and hypoxia-inducible factor-1α (HIF-1α) may serve as potential biomarkers for peripheral microcirculatory disorders in patients with systemic sclerosis
2. The serum concentration of copeptin shows a positive correlation with the severity of Raynaud's phenomenon and the occurrence of peripheral ulcers in systemic sclerosis. It is also a sensitive parameter for response to rheological treatment. The serum concentration of copeptin is a sensitive parameter for monitoring the response to rheological treatment
3. Serum hypoxia-inducible factor-1 alpha concentration is statistically higher in patients with an "active" capillaroscopic pattern of systemic sclerosis according to Cutolo's classification, compared to patients with an "early" and "late" pattern
4. Monitoring the concentrations of copeptin and hypoxia-inducible factor-1 alpha in patients with systemic sclerosis may allow the identification of individuals at high risk of severe microcirculation disorders and, consequently, the early initiation of intensive rheological treatment

4. WSTĘP

Twardzina układowa (*Systemic Sclerosis – SSc*) to autoimmunologiczna choroba tkanki łącznej charakteryzująca się postępującym włóknieniem skóry i narządów wewnętrznych. Choroba ma przewlekły, postępujący przebieg prowadzący do niepełnosprawności chorych oraz istotnego obniżenia jakości życia [1]. W złożonej patogenezie twardziny układowej wyróżnia się 3 główne procesy [2]:

- zaburzenia morfologii i funkcji naczyń mikrokrążenia
- zaburzenia immunologiczne (obecność autoprzeciwciał w surowicy i nacieków zapalnych w zajętych tkankach)
- postępujące włóknienie skóry i narządów wewnętrznych

W rozwoju twardziny układowej najwcześniejsze zmiany dotyczą uszkodzenia komórek śródbłonka i zaburzeń mikrokrążenia, a sam proces włóknienia wydaje się wtórnym do stymulacji przez cytokiny uwalniane ze śródbłonka, płytek krwi i komórek zapalnych [3]. Zaburzenia mikrokrążenie przyjmują szerokie spektrum objawów. Jedną z ich najwcześniejszych manifestacji klinicznych, która niejednokrotnie wyprzedza pojawienie się innych cech twardziny układowej nawet o kilka lat, jest objaw Raynauda, stwierdzany u ponad 95% chorych [4]. Dalsza progresja zaburzeń morfologii i czynności drobnych naczyń przyczynia się do niedokrwienia tkanek z powikłaniami w postaci owrzodzeń, martwicy i autoamputacji paliczków. Zaburzenia mikrokrążenia obwodowego prowadzą do wystąpienia bólu oraz upośledzenia sprawności pacjentów [1].

W twardzinie układowej kluczowe jest wczesne rozpoznanie oraz identyfikacja chorych z ryzykiem wystąpienia powikłań narządowych. Heterogenność objawów stanowi problem w szybkiej identyfikacji pacjentów zagrożonych szybkim postępem choroby i rozwojem ciężkich powikłań narządowych. Wczesna intensyfikacja leczenia naczyniowego i immunosupresyjnego jeszcze na etapie subklinicznym, znacząco poprawiłaby skuteczność terapii i rokowanie u pacjentów z twardziną układową [5]. Dlatego też trwają poszukiwania biomarkerów, które pozwolą na:

- monitorowanie nasilenia choroby (korelacja z włóknieniem skóry i uszkodzeniem narządów wewnętrznych)
- określenie rokowania przebiegu choroby (identyfikacja pacjentów o większym ryzyku szybkiej progresji i rozwoju ciężkich powikłań narządowych)
- ocenę odpowiedzi na leczenie (szybka identyfikacja pacjentów nieodpowiadających na zastosowaną terapię).

Ponieważ uszkodzenie mikrokrążenia występuje najwcześniej w rozwoju twardziny układowej, dlatego też zbadanie biomarkerów związane z wpływem na budowę i funkcję naczynia, wydają się być optymalnym podejściem.

Analizowane biomarkery wraz z uzasadnieniem wyboru

Kopeptyna

Kopeptyna jest C-końcową częścią pro-wazopresyny. Jej cechy, takie jak stabilność w osoczu, łatwość zmierzenia oraz dłuższy okres półtrwania niż wazopresyny sprawiają, że jest ona coraz szerzej badaną cząsteczką dla określenia aktywności układu wazopresynergicznego w organizmie [6]. Wazopresyna jest ważnym peptydem o właściwościach wazokonstrykcyjnych, który może podobnie do angiotensyny II i endoteliny, być zaangażowanym w skurcz naczyń podczas objawu Raynauda oraz uszkodzenie komórek śródbłonka [7].

Zmiany naczyniowe w twardzinie układowej obejmują nie tylko waskulopatię ale również deregulację napięcia naczyniowego [8]. Opór naczyń krwionośnych jest regulowany przez złożoną interakcję między modulacją układu współczulnego, a miejscowymi i ogólnoustrojowymi mediatorami wazoaktywnymi, takimi jak angiotensyna II, endotelina-1 i wazopresyna [9]. Angiotensyna II (Ang II) to wysoce aktywny oktapeptyd biorący udział w regulacji ciśnienia krwi. Angiotensyna II ma dwa główne podtypy receptorów, typ 1 (AT1R) i typ 2 (AT2R), należące do siedmiu błon transbłonowych obejmujących nadrodzinę receptorów sprzężonych z białkiem G [10]. Chociaż klasycznie angiotensyna II znana jest ze swojej roli w utrzymaniu homeostazy układu krążenia, obecnie uznawana jest również za mediator stanu zapalnego i zwłóknienia poprzez stymulację AT1R [11, 12]. Patofizjologiczne znaczenie zaburzeń osi angiotensyna I/angiotensyna II/angiotensyna-(1-7) zostało potwierdzone w patogenezie twardziny układowej [13]. Endotelina-1 (ET-1) jest kolejnym silnym endogennym czynnikiem obkurczającym naczynia krwionośne. Wykazano również jej rolę we włóknienie komórek naczyniowych, poprzez stymulację produkcji reaktywnych form tlenu i cytokin prozapalnych [14, 15]. Agonistyczne autoprzeciwciała skierowane przeciwko receptorom AT1 i ET-1 typu A u pacjentów z SSc są związane z większym ryzykiem śmiertelności i zajęciem narządowym [16].

Wazopresyna (AVP) jako peptyd wazoaktywny, podobnie jak angiotensyna II i endotelina-1 może brać udział w skurczu naczyń, stanach zapalnych i zwłóknieniu [17]. Do tej pory AVP był intensywnie badany w różnych patologiach układu sercowo-

naczyniowego [6] według naszej wiedzy dotychczas nie ma dostępnych danych na dotyczących układu wazopresynergicznego u pacjentów z twardziną układową.

Czynnik indukowany hipoksją-1α (Hypoxia Inducible Factor-1α; HIF-1α)

Kluczowy czynnik transkrypcyjny w odpowiedzi na przewlekłe niedotlenienie, może mieć istotne znaczenie w patogenezie chorób przebiegających z nadmiernym włóknieniem [18].

HIF-1 jest heterodimerem, złożonym z podjednostek α i β [19]. Poziom podjednostki HIF-1α związany jest ze stopniem zawartości tlenu w komórkach i w czasie hipoksji gwałtownie wzrasta. Ekspresja podjednostki HIF-1β jest konstytutywnie stabilna i niezależna od stężenia tlenu w komórce [20]. Oprócz HIF-1 istnieją dwa inne izotypy z rodziny czynników indukowanych niedotlenieniem, znane jako HIF-2 i HIF-3, które również odgrywają rolę w odpowiedziach transkrypcyjnych na niedotlenienie, w tym neowaskularyzację [21]. Jednak HIF-1 jest uważany za jeden z najważniejszych czynników indukowanych niedotlenieniem, zaangażowanych w metabolizm komórkowy, procesy naprawcze tkanek w odpowiedzi na zapalenie [21]. HIF-1 stymuluje produkcję włókien macierzy pozakomórkowej, przebudowę naczyń i nieprawidłową angiogenezę, skutkując nasileniem hipoksji [22]. Wykazano, że sygnalizacja HIF-1α jest związana z chorobami sercowo-naczyniowymi, zapalnymi i metabolicznymi [23].

Coraz więcej dowodów wskazuje, że czynnik indukowany niedotlenieniem-1α, może być zaangażowany w patogenezę chorób przebiegających z postępującym włóknieniem, takich jak twardzina układowa [18]. Polimorfizm rs12434438 genu dla HIF-1 związany jest z predyspozycją do rozwoju twardziny układowej [24]. Takagi i wsp. [25] wykazali, że genotyp AA w rs12434438 był związany z podgrupą pacjentów z SSc z ciężkim tętniczym nadciśnieniem płucnym (PAH), co sugeruje, że SNP rs12434438 może przyczyniać się do rozwoju PAH z SSc [25]. Wyniki badania przeprowadzonego przez Mao i wsp. [26] sugerują, że szlak sygnałowy HIF-1α/czynnik wzrostu śródbłonka naczyń (VEGF) może odgrywać kluczową rolę w pośredniczeniu w indukowanej hipoksją śródbłonkowej przemianie mezenchymalnej (EndMT) obserwowanej w mikrokrążeniu skórnym pacjentów z SSc. Uszkodzenie komórek śródbłonka jest kluczowym zdarzeniem wyzwalającym przebudowę naczyń, patologiczny rozrost tętniczek błony wewnętrznej, w konsekwencji okluzję naczyń krwionośnych [26]. Znaczenie przejścia śródbłonka do mezenchymalnego (EndMT) w patofizjologii zwłóknienia tkanek i waskulopatii obserwowanych w różnych chorobach z postępującym włóknieniem jest opisywane w patofizjologii twardziny układowej [27].

Znalezienie nowych biomarkerów może pomóc w wyodrębnieniu pacjentów z szybką progresją zmian naczyniowych i wysokim ryzykiem powikłań narządowych. Zmiany stężenia analizowanych związków mogą okazać się pomocne jako wskaźniki odpowiedzi na leczenie naczyniowe. Odpowiednio wczesna intensyfikacja leczenia reologicznego może zapobiec patologicznej przebudowie naczyń i spowolnić proces włóknienia [28, 29].

5. ZAŁOŻENIA I CEL PRACY:

Celem pracy była analiza zaburzeń mikrokrążenia prowadzącego do objawu Raynauda i patologii nerek u pacjentów z twardziną układową oraz poszukiwanie nowych biomarkerów związanych z uszkodzeniem naczyń w przebiegu twardziny układowej, w tym w szczególności:

1) Ocena możliwości wykorzystania kopeptyny i czynnika indukowanego hipoksją-1α (HIF-1α) jako potencjalnych biomarkerów zaburzeń mikrokrążenia w twardzinie układowej
2) Ocena potencjalnej korelacji między stężeniem kopeptyny oraz czynnika indukowanego hipoksją-1α i aktywnością twardziny układowej
3) Analiza wpływu leczenia reologicznego alprostadylem na stężenie wybranych biomarkerów zaburzeń mikrokrążenia u pacjentów z twardziną układową

6. PUBLIKACJE STANOWIĄCE PRACĘ DOKTORSKĄ

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Review Article

Renal Involvement in Systemic Sclerosis: An Update

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Keywords
Angiotensin-converting enzyme inhibitors · Hypertension · Kidney · Systemic sclerosis · Scleroderma renal crisis

Abstract
Background: Systemic sclerosis is an immune-mediated rheumatic disease characterized by vascular abnormalities, tissue fibrosis and autoimmune phenomena. ***Summary:*** Renal disease occurring in patients with systemic sclerosis may have a variable clinicopathological picture. The most specific renal condition associated with systemic sclerosis is scleroderma renal crisis, characterized by acute onset of renal failure and severe hypertension. Although the management of scleroderma renal crisis was revolutionized by the introduction of angiotensin-converting enzyme inhibitors, there is still a significant proportion of patients with poor outcomes. Therefore, research on establishing disease markers (clinical, ultrasonographical and serological) and clear diagnostic criteria, which could limit the risk of developing scleroderma renal crisis and facilitate diagnosis of this complication, is ongoing. Other forms of renal involvement in systemic sclerosis include vasculitis, an isolated reduced glomerular filtration rate in systemic sclerosis, antiphospholipid-associated nephropathy, high intrarenal arterial stiffness and proteinuria. ***Key Messages:*** Scleroderma renal crisis is the most specific and life-threatening renal presentation of systemic sclerosis, albeit with declining prevalence. In patients with scleroderma renal crisis, it is mandatory to control blood pressure early with increasing doses of angiotensin-converting enzyme inhibitors, along with other antihypertensive drugs if necessary. There is a strong association between renal involvement and patients' outcomes in systemic sclerosis; consequently, it becomes mandatory to find markers that may be used to identify patients with an especially high risk of scleroderma renal crisis.

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Introduction

Systemic sclerosis (SSc) is a chronic multisystem disease characterized by thickening of the skin and fibrosis of various internal organs [1, 2]. Although the exact pathogenesis of SSc still remains incompletely understood, vasculopathy and dysregulation of the immune system are considered to play a significant role [3].

In SSc one of the most significant, acute consequences of vasospasm and arterial damage is renal involvement. In contrast to pulmonary arterial hypertension, which is characterized by slowly progressive vasculopathy, the vascular changes in renal scleroderma usually develop rapidly due to higher values of systemic blood pressure in comparison to pulmonary pressure [4]. The renal involvement in SSc may remain subclinical until the late stages [5]. Autopsy studies reveal occult renal pathology in 60–80% of SSc patients [6]. Cannon et al. [7] found that up to 50% of asymptomatic patients have clinical markers indicative of renal dysfunction, such as proteinuria, an increased creatinine concentration, or hypertension. Various studies suggest a strong association between renal involvement and worse patient outcomes in SSc [3, 8]. In addition, a multinational SSc inception cohort study found renal crisis to be one of the predictors of early mortality in SSc patients [9].

The most studied form of renal involvement, associated with the most dramatic clinical course, is scleroderma renal crisis (SRC). The other forms of renal involvement include anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, an isolated reduced glomerular filtration rate (GFR) in SSc, antiphospholipid (aPL)-associated nephropathy, high intrarenal arterial stiffness and proteinuria (Fig. 1).

Scleroderma Renal Crisis

The most specific form of renal involvement in SSc is SRC, characterized by acute onset of renal failure and severe hypertension. On presentation, 90% of patients with SRC consistently have blood pressure levels exceeding 150/90 mm Hg and decreased renal function (≥30% reduction in estimated GFR [eGFR]). SRC occurs in 10–15% of patients with diffuse cutaneous SSc (dcSSc) and only rarely (1–2%) in limited cutaneous SSc (lcSSc) [10, 11]. SRC was a predominant cause of death in previous decades [12, 13]; however, in recent years, declining trends have been observed [14]. An analysis of 637 patients with dcSSc with a disease duration <4 years from the European League against Rheumatism Scleroderma Trials and Research (EUSTAR) cohort has shown the prevalence of SRC at an estimated 2.4% [15, 16].

Pathogenesis of SRC

The exact pathogenesis of SRC is still under investigation, but genetic and environmental factors are likely involved. The primary pathogenic process is thought to be injury to endothelial cells resulting in intimal thickening and proliferation of the renal intralobular and arcuate arteries. SRC appears histologically as "onionskin lesions" of the renal interlobular arteries [17]. Antiendothelial cell antibodies, which are capable of inducing endothelial cell apoptosis, have been detected in up to 85% of SSc patients [18]. In addition to structural changes, episodic vasospasm, named "renal Raynaud's phenomenon," contributes to renal hypoperfusion, increased renin release and juxtaglomerular hyperplasia. Hyperreninemia causes vasoconstriction and renal ischemia, which contributes to accelerated hypertension [19–21]. Endothelin-1, a peptide that plays a role in blood vessel constriction, and its receptor ET-B are overexpressed in patients with SRC [22].

Alterations in cellular and humoral immunity may both play a role in SRC pathogenesis [23–25]. SSc has been associated with activation of type 2 helper T lymphocytes, cytokine

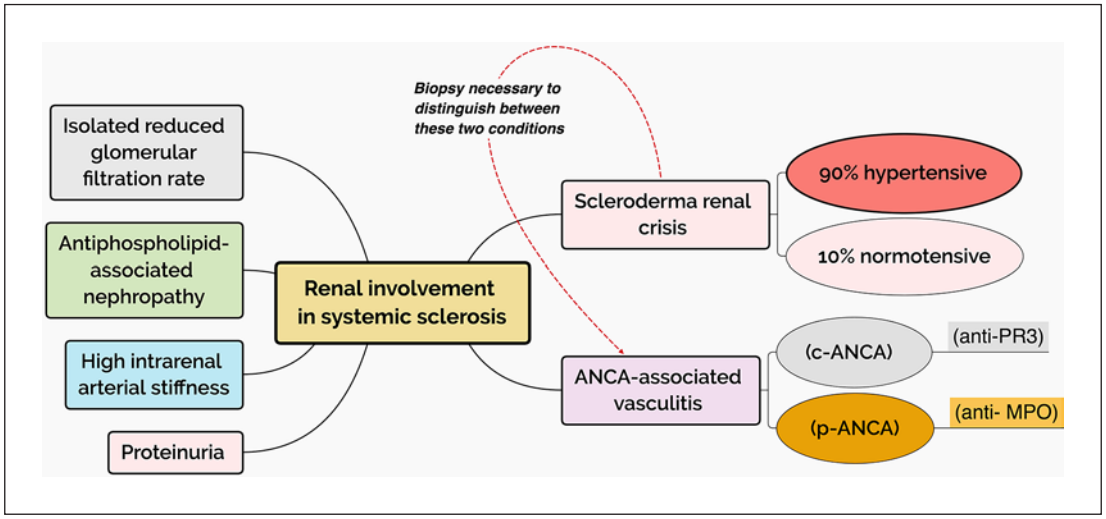

Fig. 1. The spectrum of kidney involvement in systemic sclerosis. c-ANCA, diffuse cytoplasmic staining; anti-PR3, anti-proteinase 3 antibodies; p-ANCA, perinuclear pattern; anti-MPO, anti-myeloperoxidase antibodies.

production and excess collagen accumulation, all of which participate in the development of vasculopathy [26]. The potential pathogenic role of specific autoantibodies is implied by the association between their presence and the development of SRC [27, 28]. Furthermore, the complement degradation product C4d, which is regarded as an immunologic marker of antibody-mediated rejection of renal allografts, was detected in native renal biopsies from a subset of SRC patients [29].

There is a strong relationship between SRC development and specific MHC classes, in particular HLA (human leukocyte antigen)-DRB1*0407 and HLA-DRB1*1304. Additionally, gene screening studies showed an association of SRC with genes in the complement region. Polymorphism in the endothelin ligand receptor axis, but not in the angiotensin-converting enzyme (ACE) axis, has also been associated with an increased risk of SRC [30].

CD147, also known as extracellular matrix metalloproteinase inducer, is a glycosylated membrane protein which belongs to the immunoglobulin superfamily. Yanaba et al. [31] found that an increased concentration of serum soluble CD147 was associated with the presence of SRC. Therefore, CD147 may play a role in the development of SRC, and measurement of circulating soluble CD147 may be a useful biomarker for SRC risk stratification.

Clinical Symptoms of SRC

Patients with SRC may present with headaches, hypertensive retinopathy, encephalopathy, seizures, fever and general malaise. Pulmonary edema is also common, resulting from water and salt retention due to overload and oliguria [32, 33].

Laboratory tests of patients with SRC reveal multiple abnormalities. A rapid increase in serum creatinine concentration and/or microangiopathic hemolytic anemia is associated with severe glomerular damage and with intravascular hemolysis [34]. In addition, the renin concentration is also significantly increased [7]. Urinalysis commonly demonstrates mild proteinuria and hematuria with granular casts visible on microscopy [35]. Markers of endothelial cell perturbation have been observed, including a high concentration of soluble adhesion molecules in the blood (VCAM-1, ICAM-1 and E-selectin) [36].

Ninety-nine experts evaluated a broad list of potential diagnostic criteria in order to identify key aspects of SRC [37], the results of the study were used for the development of classification criteria for SRC (Fig. 2). A consensus was reached regarding the following

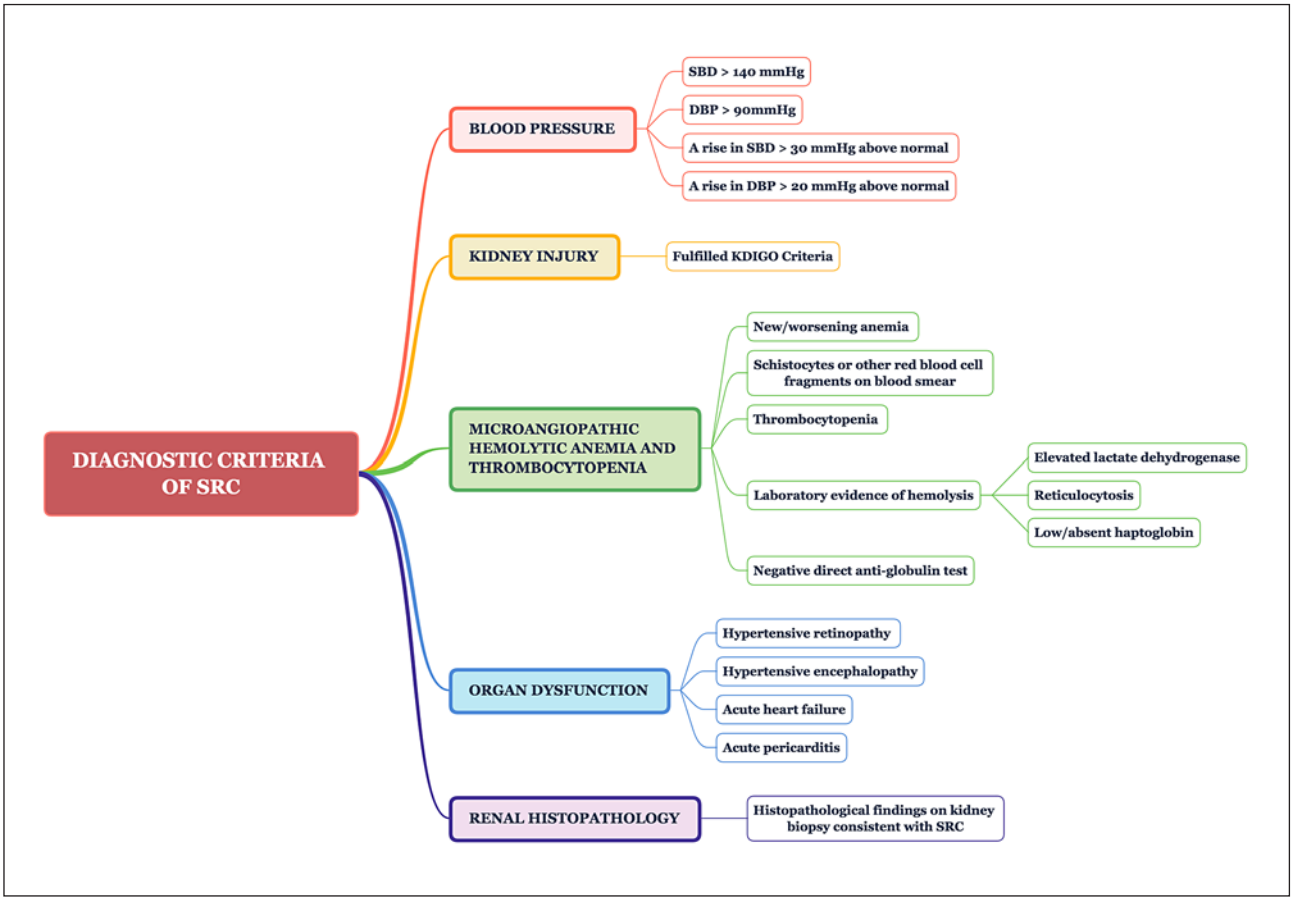

Fig. 2. Proposed diagnostic criteria for scleroderma renal crisis (SRC). SBD, systolic blood pressure (German: "systolischer Blutdruck"); DBP, diastolic blood pressure; KDIGO, Kidney Disease: Improving Global Outcomes.

aspects and may be a useful tool that summarizes valid clinical features of SRC, thus directing the diagnosis.

Blood Pressure. (1) Acute rise in blood pressure defined as one or both of the following: systolic blood pressure >140 mm Hg; diastolic blood pressure >90 mm Hg. (2) A rise in systolic blood pressure >30 mm Hg above normal and/or diastolic blood pressure >20 mm Hg above normal. (3) Blood pressure measurement should be taken twice, separated by at least a 5-min interval. (4) If blood pressure readings are discordant, repeat readings should be obtained until 2 consistent readings are obtained.

Kidney Injury. Acute kidney injury fulfilling Kidney Disease: Improving Global Outcomes (KDIGO) criteria.

Microangiopathic Hemolytic Anemia and Thrombocytopenia. (1) New or worsening anemia not due to other causes. (2) Schistocytes or other red blood cell fragments on blood smear. (3) Thrombocytopenia <100,000/μL, confirmed by manual smear. (4) Laboratory evidence of hemolysis: elevated lactate dehydrogenase, reticulocytosis, and/or low/absent haptoglobin. (5) Negative direct antiglobulin test.

Organ Dysfunction. (1) Hypertensive retinopathy confirmed by an ophthalmologist. (2) Hypertensive encephalopathy. (3) Acute heart failure. (4) Acute pericarditis.

Renal Histopathology. Histopathological findings on kidney biopsy consistent with SRC [37].

Table 1. Differential diagnosis of scleroderma renal crisis

ANCA-associated vasculitis
Malignant hypertension
Drug-induced renal injury
Complement dysregulation
Hemolytic uremic syndrome/thrombotic thrombocytopenic purpura
Transplant rejection

The proposed diagnostic criteria for SRC are presented in Figure 2.

Renal biopsy is not recommended in patients with SSc presenting with typical features of SRC, but it plays a key role in cases with atypical presentation or in doubtful cases [38–40]. SSc mostly affects the interlobular arteries, in contrast to hemolytic uremic syndrome/thrombotic thrombocytopenic purpura (HUS/TTP), which predominantly affect the glomeruli. Malignant hypertension-associated thrombotic microangiopathy may have an appearance identical to that seen in SSc, although this is more often associated with underlying arteriolonephrosclerosis. Clinical history and serological data help to distinguish these etiologies [41]. Table 1 shows the differential diagnosis of SRC.

Risk Factors for SRC Development

Rapidly progressive skin thickening appears to be the main risk factor for SRC, with most dcSSc patients developing SRC within 7.5 months to 4 years of disease onset [10, 11, 42]. According to Moinzadeh et al. [43], development of proteinuria and/or hypertension, as well as positive anti-RNA polymerase III (anti-RNAP III) antibodies are the strongest risk factors for SRC. Terras et al. [44] and Stochmal et al. [45] showed that the presence of anti-RNAP III antibodies in patients with SSc correlates with renal crisis and severe cutaneous involvement. Although an association between SRC and anti-RNAP III antibodies has already been reported in patients with dcSSc, Takada et al. [46] recently reported a case of anti-RNAP III antibody-associated SRC in a patient with lcSSc.

A number of other risk factors have been identified, for example, anemia [42], recent cardiac events, large joint contractures [47], digital pitting scars, myalgia and myopathy [38]. Shimizu et al. [48] reported a case of SRC complicated by thrombotic microangiopathy in a patient with no other risk factors after infection with influenza B virus. In regard to medications, glucocorticoids have been implicated as a major risk factor when used in doses ≥15 mg per day in the preceding 6 months [49]. It is hypothesized that high doses of corticosteroids can promote endothelial dysfunction and inhibit prostacyclin production, which in turn increase ACE activity [50]. Cyclosporin therapy is another risk factor for SRC development [51].

Treatment of SRC and Preventive Measures

The key to management of SRC is early detection and treatment with ACE inhibitors (ACE-I) [52]. The EULAR recommends ACE-I as first-line treatment, and that patients started on steroids should be carefully monitored for the development of SRC [53]. Studies have shown that if the diagnosis of SRC is delayed or if ACE-I are not used aggressively, irreversible kidney damage and death are more likely to occur [34, 54]. Early detection and aggressive treatment are also crucial to prevent other complications associated with SRC, such as hypertension, retinopathy and pulmonary edema [35]. Unfortunately, the data show that over half of SRC cases have a delay in diagnosis, and thus long-term mortality remains significant [55]. If blood pressure control remains suboptimal at the maximum tolerated doses of ACE-I, other antihypertensive drugs (calcium channel blockers as second-line and diuretics or alpha-

blockers as third-line treatment) should be added. Angiotensin II receptor blockers should be used only in patients intolerant to ACE-I [52]. There has also been recent interest in combining ACE-I with endothelin receptor blockers and agents targeting complement component 5 [54]. The target for antihypertensive treatment is to decrease systolic blood pressure by 20 mm Hg and diastolic blood pressure by 10 mm Hg per 24 h to the normal range, avoiding hypotension.

Risk factors for poor outcome in SRC include the following: a serum creatinine concentration >3 mg/dL at presentation, male gender, a delay in initiating antihypertensive treatment, inadequate blood pressure control, congestive heart failure, normotensive SRC, and renal biopsy findings showing (1) arteriolar fibrinoid necrosis, (2) severe glomerular ischemic collapse or (3) severe tubular atrophy and interstitial fibrosis [50].

In terms of preventive measures, regular blood pressure monitoring and seeking early attention when blood pressure suddenly increases [11, 14] are vital. Patients deemed at high risk for SRC may benefit from shorter follow-up intervals with particular focus on subtle changes or abnormalities in proteinuria and eGFR, intensive home blood pressure monitoring (to include 24-h ambulatory blood pressure monitors) and earlier nephrology consultation [56]. Renal function should be assessed regularly via serum creatinine concentration, eGFR and serum renin concentration, as well as via general urine analysis and 24-h proteinuria [57].

There is no evidence at present to support the use of ACE-I prophylactically, although prompt initiation of treatment remains a key point in SRC therapy. New therapeutic options are needed [14, 53].

Plasma exchange, which has been proposed for thrombotic microangiopathy, has not demonstrated efficacy and should not be recommended, with the exception of rare SRC patients who might develop thrombotic microangiopathy associated with anti-ADAMTS-13 antibodies [58]. There are currently no clinical trial data regarding the use of plasma exchange in SRC.

In severe cases of SRC with systemic complement activation and resistant to conventional treatment, eculizumab, the C5 blocker, has recently been proposed as a possible treatment option. Eculizumab is a humanized recombinant monoclonal antibody directed against complement component 5. The drug inhibits the generation of C5a and C5b-9, and thus inhibits lysis and endothelial damage [59]. The rationale for the involvement of the complement system in the pathogenesis of SRC, as well as for the usage of eculizumab, is as follows: an association of hypocomplementemia with SSc and vascular involvement, occurrence of microangiopathic hemolytic anemia in SRC, C5b-9 deposits in capillaries of SSc patients' skin biopsies and C4d deposits in renal peritubular capillaries of SSc patients with a poor renal outcome [60].

Renal Replacement Therapy and Renal Transplantation

Despite treatment, dialysis is still needed in 23% of SRC patients, and permanent dialysis in 41% of SRC patients [10]. Up to half of the cases requiring renal replacement therapy eventually come off dialysis, although this may be between 6 and 24 months after the initial SRC. Renal transplantation offers superior survival in SSc compared with long-term dialysis [61], although it should be emphasized that renal transplantation is not always possible due to the severe multiorgan involvement. In a cohort of 260 patients with SSc who underwent kidney transplantation, the overall 5-year graft survival rate was 56.7% [62]. In a report of the United Network of Organ Sharing, recurrence of disease after transplantation was 6.7% [58]. Based on the finding that cyclosporine may be responsible for acute renal failure in patients with SSc, calcineurin inhibitors are not recommended as immunosuppressants after kidney transplantation [51]. Data from the Australia and New Zealand Dialysis and Transplant (ANZDATA)

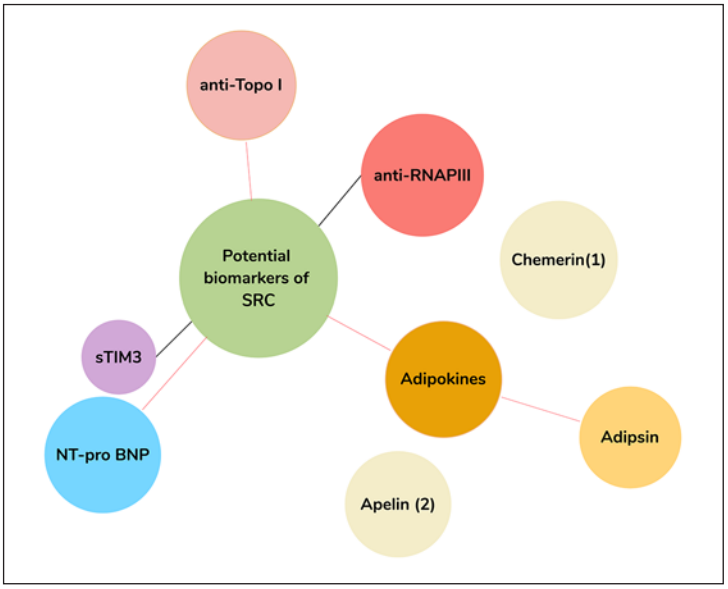

Fig. 3. Potential biomarkers of scleroderma renal crisis (SRC). (1) Promising marker of increased risk of impaired renal function in the early stage of systemic sclerosis; the role in SRC is not established. (2) Inhibits the fibrotic process in numerous organs including the kidneys, secretion in systemic sclerosis is decreased; the role in SRC is not established. anti-Topo I, anti-topoisomerase I; anti-RNAP III, anti-RNA polymerase III antibodies; sTIM3, soluble T-cell immunoglobulin and mucin domain 3; NT-proBNP, N-terminal prohormone of brain natriuretic peptide.

registry and the European Renal Association-European Dialysis and Transplant Association (ERA-EDTA) Registry confirmed that overall survival of SSc patients receiving renal replacement therapy is worse than that of patients with other causes of end-stage renal disease. However, patients with SSc have a higher rate of dialysis-independent renal function recovery [63, 64].

Antinuclear Antibodies Associated with SRC

Autoimmune serology can be used to identify patients at significant risk of SRC. Anti-RNAP III antibodies and anti-topoisomerase I (anti-Topo I/anti-Scl70) have been associated with an increased risk of developing SRC. The anti-centromere antibodies, typically seen in lcSSc, are considered "protective" against renal crisis [65]. A proportion of 16.7–24% of patients with the anti-RNAPIII antibodies develop SRC [44, 66, 67].

An Italian study [68] showed that patients with anti-RNAP I–III antibodies tend to develop SRC early in the course of the disease, while anti-Topo I-positive patients typically develop SRC later. In that study, 100% of the patients who were anti-RNAP I–III positive developed SRC within 18 months from disease onset, and in almost 30% of the patients, SRC was the presenting symptom of the disease. In the anti-Topo I-positive group, the onset of SRC occurred within 18 months of the disease in only 50% of the cases; 23% of the patients developed SRC between 18 and 48 months, and 27% >48 months after the disease onset. All anti-RNAP I–III-positive patients developed hypertensive renal crisis, while in the anti-Topo I group, 40% were diagnosed with the normotensive type of SRC. Patients with anti-Topo I antibodies tended to have higher creatinine levels and a less favorable outcome.

The available data indicate that detecting SSc-specific antibodies with a prognostic value may lead to a better risk stratification.

Other Biomarkers

Chiba et al. [69] found soluble T-cell immunoglobulin and mucin domain 3 (sTIM3) to be more frequently elevated in patients with a history of SRC. Chighizola et al. [70] identified N-terminal prohormone of brain natriuretic peptide (NT-proBNP) as a potentially useful biomarker for risk stratification of renal outcome in SRC, selectively identifying patients likely to require renal replacement therapy.

Table 2. Comparison of hypertensive and normotensive SRC

Hypertensive SRC	Normotensive SRC
90% of SRC cases	10% of SRC cases
BP levels >150/90 mm Hg	Absence of hypertension although BP is often elevated compared to the patient's baseline values; worse renal outcome and higher mortality
Nearly half of patients require renal replacement therapy 6–24 months after SRC	Earlier need for renal replacement therapy
Good response to treatment with ACE-I	ACE-I or dialysis seems to be less effective in this group

SRC, scleroderma renal crisis; BP, blood pressure; ACE-I, angiotensin-converting-enzyme inhibitor.

Recently, adipokines (cell signaling proteins secreted by adipose tissue) have attracted much attention as a cytokine family contributing to the various pathological processes of SSc. Chemerin, one of the adipokines, appears to be a promising marker of increased risk of impaired renal function in the early stage of SSc [71, 72]. Adipsin was suggested to take part in the pathogenesis of SRC due to an alternative pathway of complement activation [60]. Apelin, the secretion of which is decreased in SSc, was reported to inhibit the fibrotic process in numerous organs including the kidneys [73].

Other adipokines have been associated with organ fibrosis in SSc; however, their link with renal involvement is not fully established and requires further investigation (Fig. 3) [74].

Normotensive SRC

In approximately 10% of patients, SRC occurs in the absence of hypertension (normotensive SRC) [75–77]. Relative hypertension may be present, i.e., a significant increase in blood pressure which still remains within the normal range but is elevated compared to the patient's baseline values (e.g., 130/85 mm Hg in a young woman whose baseline value is 100/70 mm Hg) [78]. Absence of elevated blood pressure in SRC may delay its diagnosis and treatment, leading to disease progression. Therefore, any change in blood pressure or any kidney dysfunction should lead to close monitoring and additional tests. Normotensive renal crisis diagnosis is particularly challenging and requires confirmation of progressive azotemia and/or microangiopathic hemolytic anemia with thrombocytopenia. To diagnose normotensive renal crisis, an elevated serum creatinine concentration and at least one of the following should be found: proteinuria, hematuria, thrombocytopenia, hemolysis or hypertensive encephalopathy.

Normotensive SRC is associated with worse prognosis, a higher mortality rate and an earlier need for renal replacement therapy (Table 2) [11, 75, 79, 80]. The poor outcome has been, in part, attributed to subclinical renal injury leading to thrombotic microangiopathy in the setting of delayed diagnosis. Normotensive SRC is more common in patients with cardiac involvement. Based on several case reports, it was hypothesized that cardiac involvement may preclude a hypertensive response and worsen prognosis [76]. However, a study comparing 15 normotensive renal crisis patients with 116 hypertensive SRC patients did not show differences in the prevalence of cardiac involvement [75]. Significant differences that may contribute to a worse prognosis in normotensive SRC are more frequent occurrences of severe anemia, thrombocytopenia and pulmonary hemorrhage [75]. In the study by Helfrich et al. [75], 64% of the normotensive patients received prednisone ≥30 mg/day compared

Table 3. Comparison of SRC and ANCA-associated vasculitis

SRC	ANCA-associated vasculitis
Occurs mainly in dcSSc and only rarely (1–2%) in lcSSc	Occurs mainly in lcSSc
Malignant hypertension	Mild hypertension
Acute onset of renal failure and severe hypertension	Subacute presentation with progressive renal failure
Patients develop SRC within 7.5 months to 4 years of SSc onset	Typically occurs several years after SSc onset
ACE-I as the first-line treatment in SRC	Does not respond to ACE-I
Steroids (≥15 mg/day) are one of the major risk factors	Responsive to steroids

SRC, scleroderma renal crisis; ANCA, antineutrophil cytoplasmic antibody; SSc, systemic sclerosis; dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc; ACE-I, angiotensin-converting enzyme inhibitor.

with 16% of the hypertensive patients. Conversely, 52% of the hypertensive but only 7% of the normotensive patients received no corticosteroids.

Despite the lack of clinical studies, ACE-I are often used in normotensive SRC patients, but they seem to be less effective in this group of patients than in hypertensive SRC patients [81].

ANCA-Associated Vasculitis and SSc

ANCA are autoantibodies directed against enzymes localized within primary granules of neutrophils and lysosomes in monocytes and are implicated directly in the pathogenesis of small vessel vasculitis [82]. ANCA-associated vasculitis is rare in SSc, found in up to 9% of patients [83]. ANCA-associated vasculitis is a systemic necrotizing vasculitis of unknown etiology, including granulomatosis with polyangiitis (previously Wegener's granulomatosis), microscopic polyangiitis, renal limited vasculitis, and Churg-Strauss syndrome. Two major patterns of ANCA can be distinguished by indirect immunofluorescence: a diffuse cytoplasmic staining pattern (c-ANCA) mainly associated with anti-proteinase 3 (anti-PR3) antibodies, and a perinuclear pattern (p-ANCA) mainly associated with anti-myeloperoxidase (anti-MPO) antibodies. It has been postulated that scleroderma vasculopathy exacerbates the interaction of ANCA with the endothelium near the vascular pole and neutrophil activation in the glomerulus [53].

Most cases of ANCA-associated vasculitis are described as normotensive renal failure related to anti-MPO crescentic glomerulonephritis [84]. In contrast to classic SRC, the majority of such cases occur in lcSSc rather than dcSSc, and the process has a subacute presentation with progressive renal failure, mild hypertension and proteinuria. ANCA-associated vasculitis, in comparison to SRC, causes renal failure due to mononuclear cell infiltration and vessel wall destruction. These two conditions can only reliably be distinguished by histopathological examination [85]. ANCA-associated vasculitis typically occurs after several years of SSc – compared to SRC, which mainly occurs in the earlier stages of disease [86]. The main differences between SRC and ANCA-associated glomerulonephritis are shown in Table 3. Its diagnosis should be considered in any SSc patient with positive MPO antibodies and renal failure.

Isolated Reduced GFR in SSc

Clinically apparent renal involvement is uncommon in SSc. Many patients with SSc demonstrate less severe complications associated with a decreased GFR. The mechanisms of chronic and slowly progressive renal disease in SSc are still not fully elucidated [87]. Patients with SSc

with normal kidney function develop blood vessel abnormalities in the kidneys which are comparable to those seen in other organs. In most patients this damage is subclinical. Autopsy studies reveal occult renal pathology in 60–80% of patients with SSc, and almost all of these cases involve vascular abnormalities. It seems that renal involvement in SSc is primarily characterized by vascular damage and glomerular hypofiltration, which is quite different from patients with diabetic or hypertensive nephropathy, where hyperfiltration and increased glomerular capillary pressure represent the major causes of progressive renal dysfunction [6]. Impaired renal function may be present in SSc despite a normal serum creatinine concentration as serum creatinine may not be elevated until the GFR is <50% of normal [6].

There is an association between renal dysfunction and pulmonary hypertension. Campo et al. [88] recently evaluated 76 consecutive SSc patients with pulmonary arterial hypertension and found that at the time of diagnosis, 45.6% had renal dysfunction (eGFR <60 mL/min/1.73 m^2), while only 6.5% of them had had a prior episode of renal crisis. Furthermore, eGFR was a strong predictor of survival in this cohort, with eGFR values <60 mL/min/1.73 m^2 associated with a 3-fold increase in mortality. This strong association may be a reflection of pulmonary hypertension and right heart failure contributing to renal dysfunction through fluid retention and neuroendocrine activation.

aPL-Associated Nephropathy

aPL syndrome is characterized by antibodies directed against either phospholipids or plasma proteins bound to anionic phospholipids [89]. The presence of aPL is correlated with a constellation of clinical features including venous and arterial thrombosis, recurrent fetal loss and thrombocytopenia [90]. The prevalence of aPL in SSc varies up to 41% [91, 92]. Although the role of aPL antibodies in the pathogenesis and long-term outcomes of SSc is still unclear, the presence of aPL seems to be correlated with higher involvement of the skin and visceral organs [93, 94]. Wielosz et al. [95] suggest a relationship between kidney involvement and the presence of aPL antibodies in patients with SSc. Positivity for IgG aCL and IgG a-B2GPI in patients with SSc without secondary aPL syndrome seems to be connected with decreased glomerular filtration [95].

High Intrarenal Arterial Stiffness

Intrarenal vascular stiffness is measured via the renal vascular resistive index; using this method, a surge in arterial stiffness has been documented in SSc patients [96]. Doppler indices of intrarenal stiffness were previously also utilized as markers for prediction of new digital ulcer occurrence in SSc [97]. Additionally, it has been suggested that renal vascular stiffness parameters are increased in SSc patients with the presence of anti-RNAP III antibodies, which are a known risk factor for SRC development [98]. To date, little has been studied on the significance of the observed increase in intrarenal arterial stiffness in terms of patients' outcomes in long-term observation. Rosato et al. [99] observed renal function in SSc patients in a well-designed prospective trial with a mean follow-up of 4 years using several organ function assessment measures. In accordance with previous studies [96–99], the authors demonstrated increased intrarenal stiffness parameters in Doppler ultrasonography within the study group. During follow-up, all of the patients, except for 6 individuals in whom SRC occurred, had preserved normal renal function despite increased intrarenal stiffness. An important observation was that no significant differences in renal Doppler and serological parameters were observed between patients with renal complications (in whom SRC

Table 4. Renal manifestations of SSc

Renal dysfunction in SSc	Risk factors	Management
SRC	Diffuse skin disease Rapidly progressive skin disease Large joint contractures Prednisolone at a dose >15 mg/day <4 years since scleroderma onset Presence of anti-RNA polymerase antibody Proteinuria Anemia Recent cardiac events Digital pitting scars Myalgias and myopathy Cyclosporin therapy HLA-DRB1*0407 and HLA-DRB1*1304 High serum CD147 levels	ACE-I are the initial choice of therapy Monitoring BP several times per day with a target of <130/90 mm Hg Other antihypertensive medications (e.g., calcium channel blockers) as needed In case of severe renal failure and/or end-stage renal disease, consider dialysis as required Consider renal transplantation in dialysis-dependent eligible patients (usually within 2 years) No evidence to support prophylactic use of ACE-I In cases with dramatic clinical and histological severity, and in those that do not respond to conventional treatment, eculizumab has recently been proposed
Normotensive SRC	Cardiac involvement Previous treatment with glucocorticoid Absence of elevated BP may delay treatment, leading to disease progression	Earlier need for renal replacement therapy ACE-I or dialysis seems to be less effective in this group
ANCA-associated vasculitis MPO-ANCA	The majority of such cases occur with limited cutaneous SSc Typically occurs after several years of SSc Drugs, e.g., side effect of D-penicillamine treatment	Does not respond to ACE-I Initiate treatment with intravenous cyclophosphamide and corticosteroids Followed by maintenance therapy with azathioprine, methotrexate or mycophenolate Biopsy to distinguish ANCA-associated vasculitis from SRC
Isolated reduced GFR in SSc	Vasculopathy	Vasodilator therapy
Antiphospholipid-associated nephropathy	IgG aCL IgG a-B2GPI	Anticoagulants
Microalbuminuria and proteinuria	Disease duration >4 years Elevation of systolic BP	ACE-I Proteinuria >1 g/day – renal biopsy recommended
Penicillamine-associated renal disease	Up to 20% of patients treated with D-penicillamine develop membranous glomerulopathy with proteinuria	Discontinue D-penicillamine In more severe cases, steroids, plasmapheresis and immunosuppression have been required

SRC, scleroderma renal crisis; SSc, systemic sclerosis; ACE-I, angiotensin-converting enzyme inhibitor; BP, blood pressure; HLA, human leukocyte antigen; ANCA, antineutrophil cytoplasmic antibody; MPO, myeloperoxidase; GFR, glomerular filtration rate.

Table 5. Summary of key points

– SRC is the most frequent renal complication of SSc
– SRC is associated with a high risk of fatal outcome
– The key management of SRC is early detection and treatment with ACE-I
– The use of ACE-I at SSc diagnosis is associated with an increased risk of SRC
– High blood pressure is a common presenting feature of SRC
– Patients with SRC may be normotensive at presentation; this group has a poorer prognosis and higher mortality than the group of patients with hypertensive SRC
– Anti-topoisomerase I and anti-RNA polymerase III antibodies are associated with an increased risk of developing SRC
– ANCA-associated vasculitis in SSc does not respond to ACE-I but is responsive to steroids; histopathology is often required to exclude SRC
– Mild chronic renal insufficiency in SSc may result from vasculopathy and is probably underrecognized

SRC, scleroderma renal crisis; SSc, systemic sclerosis; ACE-I, angiotensin-converting enzyme inhibitors.

occurred) and those without renal complications. Doppler indices of intrarenal stiffness were significantly elevated in patients with digital ulcers. The study concluded that asymptomatic renal vasculopathy was present in SSc patients, but it did not seem to be associated with accelerated deterioration of renal function during follow-up [99].

Proteinuria in SSc

Increased excretion of urinary total protein is a classic marker of renal disease. Proteinuria and albuminuria are useful markers of vasculopathy and are known to be independent predictors of cardiovascular morbidity and mortality in patients with other vasculopathic diseases, such as diabetes and hypertension [100, 101]. Vascular kidney damage can be detected at an early stage by increased permeability of proteins passing the glomerular filtration barrier.

Seiberlich et al. [102] analyzed urine albumin, urine total protein and urine electrophoresis to assess protein excretion in 80 SSc patients and 18 healthy age- and gender-matched controls, all with normal GFRs. Increased total protein excretion was detected in 17.5% of SSc patients, and albuminuria was identified in 25%. Albuminuria correlated with disease duration >4 years and elevation of systolic blood pressure, suggesting it may be reflective of chronic vascular injury [102].

Proteinuria >1 g/day in SSc is uncommon and suggests an underlying glomerular disorder. In the context of serological features of lupus, significant proteinuria should be verified by renal biopsy [55].

In regard to SSc, epidemiological studies have identified proteinuria as a risk factor for increased mortality [102–104]. In patients with SSc and proteinuria, initiation of ACE-I therapy resulted in a significant decrease in urine protein excretion [105].

Conclusions

Renal involvement in patients with SSc may have a variable clinicopathological course (Table 4). The spectrum of kidney involvement includes the following: asymptomatic reduction of the GFR, ANCA-associated vasculitis, an isolated reduced GFR in scleroderma,

aPL-associated nephropathy, high intrarenal arterial stiffness and proteinuria. SRC is the most specific and life-threatening renal presentation of SSc; however, in recent years, declining frequencies have been observed.

In patients with established SRC, it is mandatory to control blood pressure early with increasing doses of ACE-I, along with other antihypertensive drugs if necessary. The lack of specific diagnostic criteria for SRC is an ongoing problem. A consensus of experts evaluated a broad list of potential diagnostic items in order to identify the key aspects of SRC.

Autoimmune serology – anti-RNAP III antibodies and anti-Topo I – can be used to identify patients with a higher risk of SRC. sTIM3 was also found to be frequently elevated in patients with a history of SRC. NT-proBNP has been proposed as a potentially useful biomarker in risk stratification of renal outcome in SRC, identifying patients in whom renal replacement therapy is more likely to be required. Recently, adipokines have attracted much attention as potential new biomarkers due to their contribution to the various pathological processes in SSc. There is a strong association between renal involvement and patients' outcomes in SSc. Consequently, it seems mandatory to find markers that may be used to identify patients with an especially high risk of SRC. The key points are summarized in Table 5.

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6.2. Maciejewska M., Sikora M., Maciejewski C., Alda-Malicka R., Czuwara, J., Rudnicka L., Raynaud's phenomenon with focus on systemic sclerosis. *J. Clin. Med.* 2022, 11, 2490.

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Review

Raynaud's Phenomenon with Focus on Systemic Sclerosis

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Abstract: Raynaud's phenomenon is a painful vascular condition in which abnormal vasoconstriction of the digital arteries causes blanching of the skin. The treatment approach can vary depending on the underlying cause of disease. Raynaud's phenomenon can present as a primary symptom, in which there is no evidence of underlying disease, or secondary to a range of medical conditions or therapies. Systemic sclerosis is one of the most frequent causes of secondary Raynaud's phenomenon; its appearance may occur long before other signs and symptoms. Timely, accurate identification of secondary Raynaud's phenomenon may accelerate a final diagnosis and positively alter prognosis. Capillaroscopy is fundamental in the diagnosis and differentiation of primary and secondary Raynaud's phenomenon. It is helpful in the very early stages of systemic sclerosis, along with its role in disease monitoring. An extensive range of pharmacotherapies with various routes of administration are available for Raynaud's phenomenon but a standardized therapeutic plan is still lacking. This review provides insight into recent advances in the understanding of Raynaud's phenomenon pathophysiology, diagnostic methods, and treatment approaches.

Keywords: Raynaud's phenomenon; microcirculation; systemic sclerosis; vasculopathy; capillaroscopy; iloprost; alprostadyl; sulodexide

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1. Introduction

Raynaud's phenomenon (RP) is defined as intermittent, excessive vasoconstriction of the microvasculature, triggered by cold exposure or emotional stress [1]. The classic clinical picture involves changes in skin colour from white (ischemia), to blue (cyanosis), and red (reperfusion). These changes are associated with a significant burden of pain and hand-related disability [2]. Raynaud's phenomenon most often occurs in the fingers/toes. Less commonly, the nose, tongue, nipples, and pinnae of the ears may be involved [3].

Raynaud's phenomenon can be subdivided into primary (idiopathic) and secondary forms [Table 1]. Primary Raynaud's phenomenon (PRP) has an estimated prevalence of 5% in the general population, and most often occurs in young women [3]. Both forms of Raynaud's phenomenon are more common in cold climates [4].

Patients with PRP have a younger age of onset (usually between the age of 15 and 30) than those with secondary Raynaud's phenomenon (SRP), and the thumb is usually not involved [5]. The latest diagnostic criteria for PRP are: history of episodic, acral, bi- or triphasic colour change; normal nailfold capillaries; antinuclear antibodies (ANAs) titer < 1:40 (i.e., negative); no association with underlying systemic disease; and no history of collagen vascular disease [6].

A large population-based cohort study revealed that low body weight and previous involuntary weight loss are significantly associated with an increased risk of RP in both men and women [7].

Symptoms of SRP depend on the frequency, severity, and duration of blood vessel spasm. Episodes usually last minutes but may last several hours, potentially causing digital ulceration, irreversible ischemia, necrosis, and secondary infection. Consequently, timely and accurate recognition of SRP is crucial as it alters patient management and prognosis [4].

Table 1. Differences between primary and secondary Raynaud's phenomenon [3,5,6,8,9]; ANA—antinuclear antibodies.

Raynaud's Phenomenon	Primary	Secondary
Age at Onset	usually between 15 and 30 years	over the age of 40
ANA	negative or low titre	often positive
Change of Microcirculation	functional vascular abnormalities	functional and structural microvascular changes
Pain or Paresthesia	rare	often
Capillaroscopy	normal capillaroscopic pattern	abnormal capillaroscopic pattern
Course	complete reversibility of episodic digital ischemia	can result in digital ulceration, irreversible ischemia and necrosis
Peripheral Pulses	strong and symmetrical	dependent
Swollen ("puffy") fingers	no	yes
Digital Ulcers	no	common

Secondary causes of RP (Figure 1) include various autoimmune connective tissue disorders—systemic sclerosis (SSc), systemic lupus erythematosus (SLE), Sjögren's syndrome, idiopathic inflammatory myopathies, antisynthetase syndrome (ASyS), thoracic outlet syndrome; cervical rib, embolic or thrombotic events; vibration-induced trauma; and multiple different medications [2,10,11]—the most relevant being β-adrenoceptor blockers, vinyl chloride, interferons, and chemotherapy [12,13]. Raynaud's phenomenon frequently represents the initial manifestation in patients who have mixed connective tissue disease (MCTD). It is the cutaneous symptom of a systemic vasculopathy that is characterized by intimal fibrosis and blood vessel obliteration that frequently leads to visceral involvement. Raynaud's phenomenon appears in 18–46% of patients with systemic lupus erythematosus [14,15]. Looking for signs of arthritis or vasculitis, as well as a number of laboratory tests (Table 2), may separate them. Complete blood count may reveal a normocytic anaemia, suggesting chronic disease or kidney failure. Blood tests for urea and electrolytes may reveal kidney impairment. Tests for rheumatoid factor, erythrocyte sedimentation rate, C-reactive protein, and autoantibody screening may reveal specific causative illnesses or an inflammatory process. Thyroid function tests may reveal hypothyroidism [16,17].

Systemic sclerosis is an autoimmune disorder characterized by inflammation, fibrosis, and microvasculopathy. It results in potentially widespread fibrosis and vascular abnormalities, which can affect the skin, lungs, heart, gastrointestinal tract and kidneys. The uncontrolled fibrosis of the skin and internal organs in systemic sclerosis leads to severe and sometimes life-threatening complications.

The underlying mechanisms are complex and remain largely unknown [18,19]. Definitive diagnosis is made with fulfilment of the 2013 European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR) classification criteria [20]. Over the past 15 years, efforts have been made towards early diagnosis [21]. Almost all individuals with SSc have detectable circulating antibodies against nuclear proteins and different SSc phenotypes are strongly associated with the different antibody types [22]. Several forms of the disease have been esteemed. Diffuse cutaneous systemic sclerosis (dcSSc) is characterized by the quickest course, with internal organ involvement already at early disease stage, and poor prognosis [23]. Skin thickening confined to sites above the elbows or knees is classified as limited cutaneous SSc (lsSSc). Around 20% of patients with lcSSc may present with features of SSc as a component of the overlap syndrome [24,25].

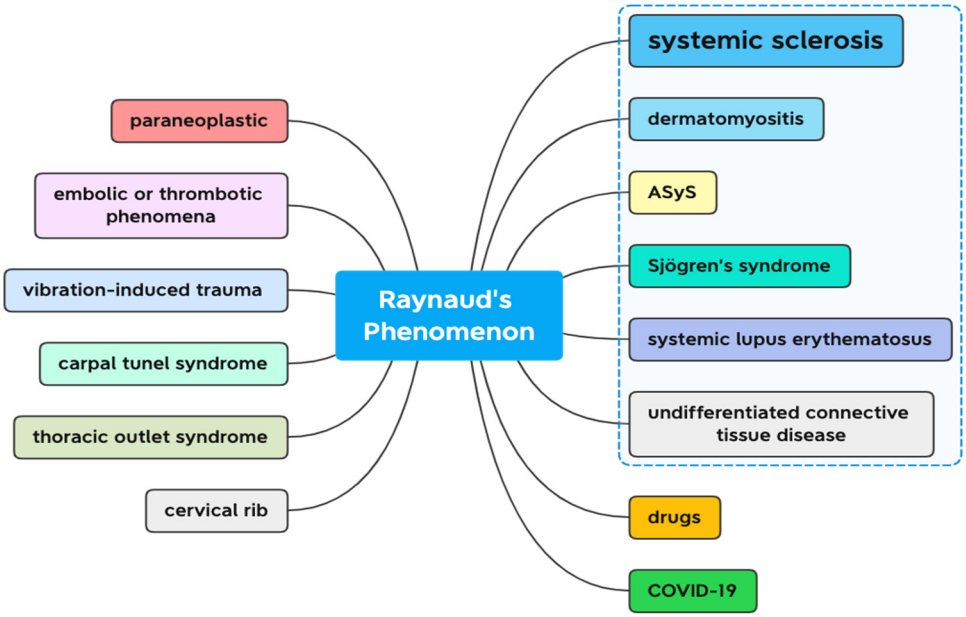

Figure 1. Secondary causes of Raynaud's phenomenon. Blue dashed line—connective tissue diseases; ASyS—antisynthetase syndrome.

Table 2. Raynaud's phenomenon—proposed laboratory tests [16,17]. TPO—thyroid peroxidase; TG—thyroglobulin; ANA—antinuclear antibody.

Raynaud's Phenomenon—Proposed Laboratory Tests

• Complete blood count
• Erythrocyte sedimentation rate
• Thyroid function tests:
  ▪ Thyroid-stimulating hormone (TSH)
  ▪ Thyroxine (T4)
  ▪ TPO antibodies
  ▪ TG antibodies
• Anti-HCV
• Rheumatoid factor
• C-reactive protein
• Cryoglobulins
• Creatinine
• Urea
• ANA test
• Protein electrophoresis
• Urinalysis

Almost all SSc patients suffer from RP, typically the initial manifestation of disease and may precede the involvement of other organs by many years, especially in lcSSc [26]. There are many areas in which RP initiates considerable disease-related morbidity in SSc patients, including impaired hand function, pain, reduced social engagement, diminished body image, increased dependence on others, and reduced quality of life [27]. Though rare, several paraneoplastic causes of RP have been identified secondary to malignancies of the lung, breast, uterus, and ovaries [28,29].

RP has additionally been reported as a side effect of biological agents—for example interferon, radiotherapy, and chemotherapeutic agents—particularly bleomycin (alone or combined with vinca alkaloids or cisplatin) and cisplatin (combined with other chemotherapy agents); beta-adrenergic blocking agents may also provoke paroxysmal vasospasm of small vessels [6].

Kim et al. reported a case of RP in a 70-year-old woman, with no history of connective tissue disease, secondary to pembrolizumab therapy for gallbladder cancer [30]. A further case report on the development of IL-17A antagonist (secukinumab)-related RP in a 35-year-old female patient with ankylosing spondylitis has also been described [31]. Additionally, Bouaziz et al. described a patient with proven COVID-19 infection presenting with RP and chilblain appearance of the hands [32].

2. Pathophysiology

The pathophysiological mechanisms behind RP are not entirely understood; generally, it is characterized by excessive vasoconstriction of the digital arteries, precapillary arterioles, and cutaneous arteriovenous anastomoses [33].

In PRP, vasospasm of the digital and cutaneous vessels is believed to occur as a consequence of an increased alpha$_{2c}$-adrenergic response, and does not result in vascular pathology [34]. Ascherman et al. put forward an autoimmune etiology, proposing cytokeratin 10 (K10) as a potential autoantigen. Their study on mice showed that anti-K10 antibodies can mediate ischemia, similar to that seen in primary Raynaud's Phenomenon [35].

In SRP, affected endothelial cells exhibit amplified exocytosis of endothelin-1 and ultra-large von Willebrand factor (ULVWF), which contribute, respectively, to increased vasospasm and capillary thrombosis. In addition, it is believed that increased transforming growth factor β (TGFβ), endothelin-1, cytokines, and angiotensin II drive the process of myofibroblast proliferation, vascular fibrosis and dropout in SSc patients [34]. Nitric oxide (NO) has a complex role in the disease process [36]. A decrease in endothelial formation of NO results in diminished vascular relaxation and extended vasoconstriction. Conversely, overproduction of NO leads to increased generation of reactive oxygen species and plays a pathogenic role in fibrosis.

Gualtierotti et al. found that markers of endothelial damage are regularly elevated in patients with PRP at their first assessment, even when there are no capillaroscopic abnormalities or autoantibodies detectable. They are particularly increased in patients with very early SSc. The plasma concentration of tissue-type plasminogen activator (t-PA) and von Willebrand factor (vWF)—two markers of endothelial damage, as well as interleukin-6 (IL-6)—a pro-inflammatory cytokine, were evaluated. After a 36-month follow-up, those with higher basal concentrations of markers of endothelial damage had developed connective tissue disease. Von Willebrand factor analysis showed clear differences between primary and secondary RP patients. These findings suggest that markers of endothelial damage are elevated in RP patients who go on to develop SSc or other connective tissue diseases, even in the absence of capillaroscopic abnormalities [37].

A recent study by Taher et al. demonstrated use of a non-invasive NO-dependent method to identify peripheral microvascular endothelial dysfunction in patients with SRP. The association between SRP and microvascular peripheral endothelial dysfunction was also significant after adjusting for confounding variables, including conventional risk factors for cardiovascular disease, and vasoactive medications. This also remained significant in women after stratifying only by sex. It was emphasized that detection of microvascular peripheral endothelial dysfunction at an early stage could help to identify individuals with SRP who are at risk of developing connective tissue disease, as well as cardiovascular disease. Early detection could additionally indicate who may benefit from frequent screening, prompt initiation of preventative treatments, and modification of risk factors [38].

3. Genetics

A genetic predisposition for RP has been demonstrated in two studies demonstrating greater concordance amongst monozygotic than dizygotic twins. Heritability for RP is reported to be 55–64% [39,40].

Polymorphisms in various genes encoding ion channels or vasoactive agents have been hypothesized to result in the RP phenotype. It is suggested that genetic variation

in temperature-responsive or vasospastic genes may underlie RP manifestation. Several studies have investigated candidate genes that could potentially regulate vascular reactivity [41,42]. Munir et al. aimed to evaluate the association between RP and single nucleotide polymorphisms (SNPs). Temperature-sensing receptor channels called thermo-sensitive transient receptor potential (TRP) ion channels include TRPA1 and TRPM8. These are cold-sensing and have been proposed to mediate cold-induced vascular responses in skin in vivo. This is linked, at least in part, to the expression of these channels on perivascular sensory nerves [41]. Calcitonin-related polypeptides, alpha and beta (CALCA, CALCB), encode the peptide hormones calcitonin, calcitonin gene-related peptide, and katacalcin by tissue-specific alternative RNA splicing of gene transcripts and cleavage of inactive precursor proteins. Calcitonin is involved in the regulation of calcium levels and phosphorus metabolism. Calcitonin gene-related peptide functions as a vasodilator [43]. NO derived from neuronal nitric oxide synthase (nNOS) facilitates the restorative vasodilator response after cold exposure; thus, the gene encoding nNOS (NOS1) has also been investigated [41]. Munir et al. found that one polymorphic variant within the NOS1 gene was significantly associated with RP in the general population [42].

4. Diagnosis

A detailed medical history, laboratory tests, and nailfold capillaroscopy form the basis of RP diagnosis [17]. Follow-up nailfold capillaroscopy should be performed every 12 months in patients with significant nailfold videocapillaroscopy disturbances present at baseline [44]. Laboratory investigations should comprise a full blood count, inflammatory markers, thyroid function, and ANA testing by indirect immunofluorescence (accompanied by ELISA or solid-phase immunoassays to determine antigen specificities where possible). A negative ANA with cytoplasmic stain could indicate anti-synthetase antibodies, such as anti-Jo-1, or rarer SSc-specific autoantibodies such as anti-eukaryotic initiation factor 2B autoantibodies (anti-EIF2B) [45].

5. Capillaroscopy

Nailfold capillaroscopy is a simple, non-invasive technique that allows both qualitative and quantitative evaluation of the microcirculation, thus enabling early detection of abnormalities. At the nailfold, capillaries are positioned parallel to the surface of the skin, allowing full morphological assessment [46,47]. Among the most important indications for capillaroscopy are the differential diagnosis of primary and secondary RP.

Capillaroscopy is included in the 2013 American College of Rheumatology (ACR)/Eur opean League Against Rheumatism (EULAR) recommendations [20]. It is considered a key investigation in both the very early phases of the disease, and in monitoring disease progression (Figure 2). Cutolo et al. proposed three progressive capillaroscopic patterns in SSc—'early', 'active', and 'late'. The 'early' pattern is defined as the presence of a few giant capillaries, single microhemorrhages, and preservation of capillary architecture without capillary loss. 'Active' presents as numerous giant capillaries and microhemorrhages, mild disturbance of the capillary architecture and moderate capillary loss. The 'late' pattern is characterized by severe capillary loss with extensive avascular areas, disorganization of the capillary architecture and ramified/bushy capillaries [48].

A clinical expert-based, fast track decision algorithm was developed to facilitate differentiation of a "non-scleroderma pattern" from a "scleroderma pattern" on capillaroscopic images. The algorithm demonstrated excellent reliability when used by capillaroscopists with varied expertise levels compared to principal experts, and corroborated with external validation [49,50].

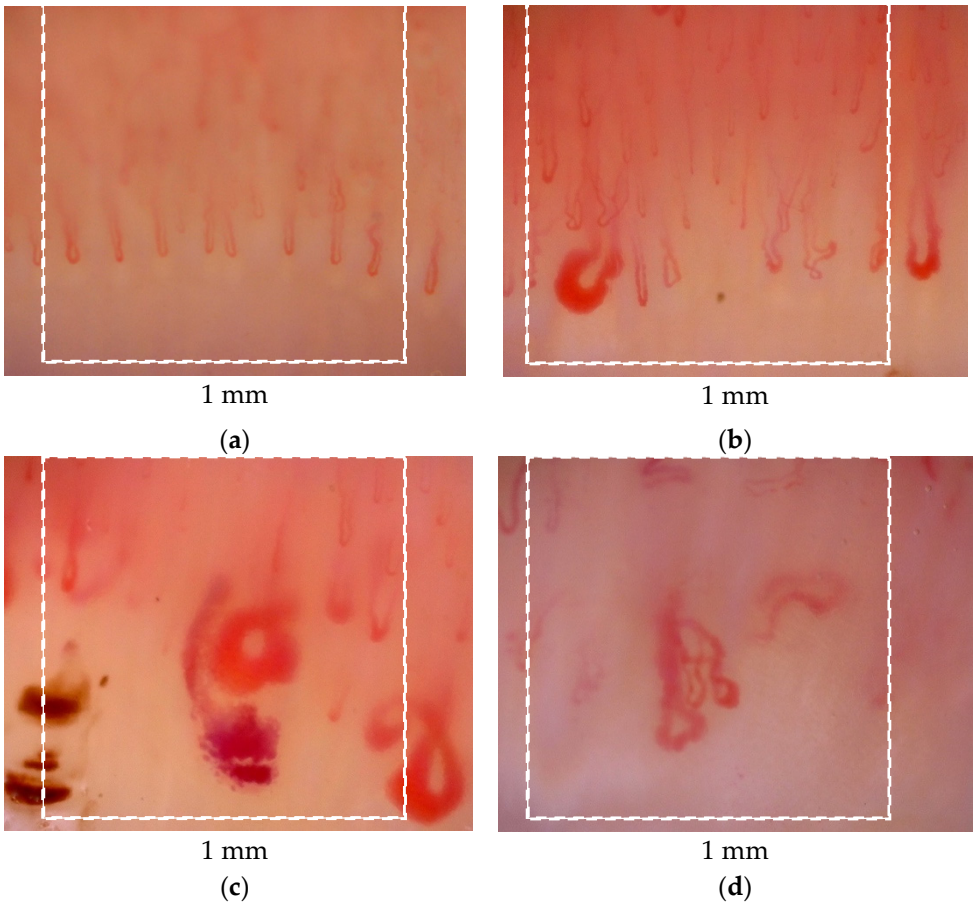

Figure 2. Nailfold videocapillaroscopic (×200) patterns of microangiopathy: (**a**)—normal; capillaroscopic characteristics: density—normal, 8 capillaries in 1 linear mm; dimension: within normal limits; morphology: normal shapes of capillaries; haemorrhages: absent (**b**)—early; capillaroscopic characteristics: density—7 capillaries in 1 linear mm; dimension: presence of giants (homogeneous enlargement of all three limbs of the capillary with the diameter ≥50 μm); morphology: hairpin shaped capillaries; haemorrhages: absent (**c**)—active; capillaroscopic characteristics: density—lowered, 4 capillaries in 1 linear mm; dimension: presence of giants (homogeneous enlargement of all three limbs of the capillary with the diameter ≥50 μm); morphology: presence of abnormally shaped capillary; haemorrhages: present (**d**)—late capillaroscopic characteristics: density—lowered, 2 capillaries in 1 linear mm; dimension: not measured because of presence of abnormal shape; morphology: presence of abnormally shaped capillary; haemorrhages: absent.

In addition capillaroscopic changes have been observed in dermatomyositis, polymyositis, antiphospholipid syndrome, Sjogren's syndrome, and systemic lupus erythematosus [51]. Dermatomyositis pattern, often associated with aspects of the SSc pattern, includes the presence of two or more of the following findings in at least two nail folds: enlargement of capillary loops, loss of capillaries, disorganization of the normal distribution of capillaries, 'budding' ('bushy') capillaries, twisted enlarged capillaries, and capillary haemorrhages (extravasates) [51,52]. Characteristic systemic lupus erythematosus pattern includes morphological alterations of capillary loops, venular visibility and sludging of blood with variability in capillary loop length [53]. Capillaroscopic abnormalities in SS ranged from non-specific findings (crossed capillaries) to more specific findings (confluent haemorrhages and pericapillary haemorrhages) or SSc-type findings [54,55]. Multiple hemorrhages from normal-shaped capillaries, which appear parallel/linear and arranged perpendicularly to the nailfold bed, are called "comb-like" hemorrhages and are suggestive of antiphospholipid syndrome [56].

It has been shown that the ability to detect capillary abnormalities increases as the number of fingers examined increases. Sensitivities ranged from 31.7% to 46.6% for only one finger (right middle and left ring finger, respectively), 59.8% for both ring fingers, 66.7% for a four-finger combination (both ring and middle fingers) and 74.6% for the eight-finger standard. In order to achieve the most accurate assessment during routine capillaroscopic examination, all eight nailbeds should be examined omitting the thumbs, where it is more difficult to visualize and classify capillaries [57,58]. It should be noted that in a time pressured scenario, the best two-finger combination to detect capillary abnormalities is both ring fingers [58].

Nailfold videocapillaroscopy is the standard, although a handheld dermatoscope or an ophthalmoscope may also be used as screening tools [50]. The nailfold videocapillaroscopy technique with 200× magnification, capturing at least two adjacent fields of 1 mm in the middle of the nailfold finger, is the standard capillaroscopic technique to perform nailfold capillaroscopy [50].

Ideally all dermatology specialists should have access to videocapillaroscopy; a pragmatic solution for practitioners may be to have a low-cost capillaroscopy system. Technologies using a smartphone camera could help to improve availability to nailfold capillaroscopy whilst still providing accurate results [59]. Research regarding automated measurement of capillaroscopic characteristics is currently under way and holds promise as an objective clinical outcome measure [50]. Interestingly, a consensus-based assessment of dermatoscopy versus nailfold videocapillaroscopy by a European League against Rheumatism study group revealed tenuous promise for dermatoscopy as a tool for the initial screening of nailfold capillaries in RP. However, as perhaps expected, dermatoscopy is less sensitive, but more specific, in regard to detecting abnormalities, compared with videocapillaroscopy [60].

Qualitative analysis is subjective, and quantitative analysis is time-consuming when done manually. A study performed by Cutolo et al. accomplished validation of fully automated AUTOCAPI software for measuring the absolute capillary number over 1 linear/mm in NVC images. The software was subsequently optimized to assess capillary number in the shortest possible time and with the lowest possible error, in both healthy subjects, and those with SSc [61].

6. Laser Doppler Flowmetry

Laser Doppler flowmetry (LDF) is a semi-quantitative imaging technique useful for studying the nitric oxide endothelial-dependent vascular response and axon reflex-mediated vasodilation. Impaired regulation of NO vascular tone has been described in patients with SSc-associated RP when compared to those with PRP and healthy controls [62,63]. Laser Doppler flowmetry has been proposed as a method for evaluating blood perfusion of the skin. This is a functional assessment of the vessels of the skin, involving the deeper dermal vessels in addition to the capillaries [64].

Melsen et al. completed a systematic review evaluating the use of LDF, describing the results of quality reports on assessment of the skin's microcirculatory flow at the level of the fingertip in SSc patients, and investigating the validation status of LDF as an outcome measure. The systematic review highlights the very preliminary validation status of LDF in the assessment of the microcirculatory flow in SSc [65].

In a study performed by Gregorczyk-Maga et al., LDF was used to investigate oral capillary flow in PRP patients who habitually have dysfunction in the microcirculation of the oral mucosa and who often have lesions in the oral cavity [66].

Time to postocclusive peak blood flow measured by LDF is an extremely accurate test for distinguishing patients with PRP from healthy controls [67].

An additional study performed by Waszczykowska et al. presented the suitability of LDF for assessment of the degree of microangiopathy present in SSc patients. Assessment of the skin perfusion value in SSc patients should on the basis of parameters obtained during microcirculation challenge tests [68].

7. Thermography

Thermal imaging is an indirect method that makes use of a thermal camera to image skin temperature and demonstrate underlying blood flow [69]. Thermal imaging has been used to evaluate RP in several studies; the response to lower temperatures was able to differentiate between PRP and RP secondary to SSc [70].

Patients with SSc-related RP have been found to have structural changes in the digital arteries and microcirculation with a decrease in baseline blood flow. This typically does not return to normal after a cold challenge with rewarming, in direct contrast to primary RP, in which the fingers classically rewarm [71].

Measurements made by mobile phone thermography compared favorably with those made by standard thermography, paving the way for ambulatory monitoring in non-controlled environments; this will enable further assessments to increase the understanding of RP episodes [69]. Infrared thermography may additionally be a method of verification in Raynaud's Phenomenon [72].

8. Laser Speckle Contrast Analysis (LASCA)

Laser speckle contrast analysis (LASCA) is a tool used to investigate variations in peripheral blood perfusion during long-term follow-up and can safely monitor the evolution of digital ulcers in SSc patients [73].

LASCA can quantify blood flow over a defined area and is based on the concept that when laser light illuminates a tissue it forms a speckle pattern. Variations in this pattern are analyzed by dedicated software—static areas demonstrate a stationary speckle pattern, in contrast with mobile objects—such as red blood cells—that cause the speckle pattern to fluctuate and appear blurred. The amount of blurring (contrast) is analyzed and thus interpreted as blood perfusion [74].

The pilot study completed by Ruaro et al. determined that the hand blood perfusion, as evaluated by LASCA, was lower in PRP than in SSc patients with the "early" nailfold videocapillaroscopy microangiopathy pattern [75].

9. Treatment

Lifestyle modifications are essential in all patients with RP [8]. Patients' education is an important aspect of disease management and patients' support organizations provide them with valuable education on the topic [76]. The first line of the treatment is based on avoiding triggering factors such as: exposure to cold, sudden changes of temperature, stress, cigarette smoke, and infections [34]. Patients should dress warmly (including warm gloves and socks). A number of different types of gloves have been proposed for patients with RP to reduce the risk of attacks, including battery-heated and specifically ceramic-impregnated gloves [77]. During the vasospasm, it is advised that one should place one's hands under warm running water or to rub one hand against the other to intensify blood flow [78]. Because stress may trigger an attack, learning to recognize and avoid stressful situations may help control the number of attacks. Exercise can improve circulation, among other health benefits. Avoidance of repeated trauma to the fingertips by all patients with RP and avoidance of vibrating tools utilization by patients with vibration-induced RP has to be underlined [79]. Patients should be counselled regarding the critical importance of smoking cessation as nicotine enhances vasoconstriction [80]. Certain medications such as beta-blockers, ergotamine, or sumatriptan, and some types of chemotherapy, specifically, cisplatin and bleomycin, were most likely to induce the Raynaud's phenomenon. If possible, alternative therapies that do not alter peripheral blood flow should be considered [12]. In most cases of primary Raynaud's phenomenon, lifestyle modifications may be sufficient to control the symptoms [17,81].

Pharmacological treatment is required when adaptive measures to avoid cold exposure are ineffective. RP reflects excessive vasoconstriction; thus, vasodilator therapy—particularly targeted to the cutaneous circulation—is a major focus. Patients with connective

tissue disease-associated RP, SSc in particular, may progress to tissue injury; hence, drug treatment often needs to be more 'aggressive' to prevent/minimize tissue loss [Table 3].

Table 3. Pharmacotherapy options in management of Raynaud's phenomenon. A-level recommendation is based on consistent and good-quality patient-oriented evidence; B-level recommendation is based on inconsistent or limited-quality patient-oriented evidence; C-level recommendation is based on consensus, usual practice, opinion, disease-oriented evidence, or case series for studies of diagnosis, treatment, prevention, or screening.

Group of Drugs	Medication	Dose	Strength of Recommendation
Calcium Channel Antagonists	nifedipine	10–20 mg 3× daily or extended-release tablets	A
	nifedypine SR	30–120 mg daily	
Phosphodiesterase Type 5 Inhibitors	sildenafil	50–100 mg 2× daily (the suggested starting dose is 12.5 mg/day, to be increased gradually depending on tolerability)	A
Prostaglandin Analogs	alprostadil (*i.v.* infusions)	pulses of 20–60 mg every 4–6 weeks	A
	iloprost (*i.v.* infusions/*p.o.*)	0.5–3 ng/kg/min (*i.v.*) for 3–5 consecutive days every 6–8 weeks or 50–150 µg 2× daily (*p.o.*)	A
	epoprostenol (*i.v.* infusions)	2 ng/kg/min in intermittent infusions of 5 to 6 hours' duration	A
First-in-class Guanylate Cyclase Stimulator	riociguat	2 mg single oral dose	C
Selective Serotonin Reuptake Inhibitors (SSRIs)	fluoxetine	20 mg/day	C
Endothelin Receptor Antagonists	bosentan	125 mg twice a day following initial dosage of 62.5 mg twice a day	C
Angiotensin II receptor blockers	losartan	25 mg once daily to 100 mg once daily	C
Mixture of glycosaminoglycans composed of dermatan sulfate and fast moving heparin	sulodexide	3–4 day cycle of intravenous sulodexide, at 600 LSU twice a day every 4–6 weeks	C
3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors	atorvastatin	40 mg/day	C
Botulinum toxin	type A	Dose dependent vasodilation with 10–100 units injections	C
Topical vasodilators	Nifedipine	10% nifedipine cream	C
	Nitroglycerin	10% nitroglycerin gel	
	sildenafil	5% sildenafil cream	
Surgical treatment	sympathectomy/arterial reconstruction		C

10. Calcium Channel Blockers

Calcium channel blockers (CCBs) are generally considered to be the first-line pharmacotherapeutic treatment of PRP, and are the group of drugs which have been most extensively researched. According to the 2017 update of the European League against Rheumatism (EULAR) recommendations for the treatment of SSc, oral therapies with CCBs are strongly recommended (strength of recommendation A) [16,82]. CCBs are currently the most frequently prescribed drug for PRP. Nifedipine and amlodipine are considered to be the most effective agents, blocking calcium channels located in the cell membranes of vascular smooth muscle and cardiac muscle. Consequently, calcium ion entry into cells is inhibited, resulting in blood vessel relaxation and improved blood supply to tissues [83].

Doses should be adjusted depending on individual tolerance, with particular caution advised in patients with low arterial blood pressure [84].

A meta-analysis of randomized clinical trials concluded that CCBs are only somewhat effective at reducing the frequency of Raynaud's attacks in PRP [42,85]. Whereas other studies suggest that CCBs may be effective at decreasing the severity of attacks, pain and disability associated with RP [83].

11. Phosphodiesterase-5 (PDE-5) Inhibitors

Phosphodiesterase-5 (PDE-5) inhibitors are commonly used as a second-line systemic agent to manage RP resistant to CCBs. Inhibition of PDE-5 activity allows accumulation of cGMP within endothelial cells, which alters the cellular response to prostacyclin or nitric oxide, and in turn dilates blood vessels [86].

A 2013 meta-analysis of six randomized controlled trials including 296 SRP patients revealed a significant, moderate effect on the clinical severity, duration, and frequency of attacks [87]. Additionally, a significant decrease in the number of digital ulcers in SSc patients with RP was found in a randomized, placebo-controlled study in patients receiving sildenafil compared to a placebo [88].

Adverse effects of these PDE-5 inhibitors include flushing, headaches, and dizziness. Less common side effects include hypotension, arrhythmias, cerebral vascular accidents, and vision changes [89].

12. Prostaglandin Analogs

While oral prostaglandins have not shown any benefit in RP, prostacyclin analogs administered intravenously exhibit a strong vasodilative effect which considerably improves the clinical condition, particularly among patients with ulcers and erosions [90].

Iloprost is a synthetic analogue of prostacyclin (PGI2), with vasodilatory and antiplatelet effects; however, it is more stable than PGI2, has a longer half-life (20 to 30 min) and better solubility [91]. Iloprost activates PGI2 receptors, thus stimulating adenylate cyclase to generate cyclic adenosine monophosphate (cAMP). PGI2 receptors inhibit vascular smooth muscle constriction and platelet aggregation. They are also expressed on endothelial cells, where they initiate multiple protective effects, including amplification of endothelial adherens junctions and decreased monolayer permeability [92]. Iloprost infusions are frequently recommended as second-line treatment after CCBs and are the first-line therapeutic choice for digital ulcerations and critical ischemia [21,93]. A meta-analysis determined that the use of iloprost in critical limb ischemia was effective in improving ulcer healing, relieving pain, and reducing the need for amputations [94]. In cases of pre-existing digital ulcerations, iloprost promotes healing and reduces the incidence of new ulcerations [16]. Three further studies described an improvement in nailfold microvascularization following iloprost treatment [95].

In 2017, the EUSTAR recommendations allocated intravenous iloprost a Grade A recommendation for management of severe SSc-related RP attacks and for digital ulcer treatment [16]. However, in the recommendations, the dosing and therapeutic regimen was not specified. The absence of an accepted regimen is a major impediment to the administration of iloprost in SSc. According to the Delphi concensus, intravenous iloprost can be useful in RP that is severe or refractory to CCB and PDE-5i. To control symptoms, it is recommended that iloprost be administered 1–3 days every month. Dosing should be determined according to the tolerance, starting from 0.5 up to 3.0 ng/kg/min. To achieve a lasting effect, infusions must be repeated regularly [16,96,97]. Interestingly, the pharmacological actions may persist longer than suggested by the pharmacokinetic profile (i.e., weeks to months) [98].

Currently, intravenous iloprost is available in several countries only for RP secondary to SSc for a duration of 3–5 days. For RP and digital ulcer healing, expert consensus proposes a regimen of 1–3 days per month, with 1 day per month for DU prevention. These recommendations allow clinicians some scope on how to personalize intravenous

iloprost therapy according to patients' needs [93]. However, although these suggestions are supported by an expert group for use in a clinical setting, it would be necessary to formally validate the recommendations in future clinical trials.

As iloprost is not available in some countries, alprostadil (a combination of prostaglandin E1 with a-cyclodextrin in a 1:1) has been found to be an effective alternative for SRP [99]. Alprostadil is primarily used to maintain patency of the ductus arteriosus, and also has mild pulmonary vasodilatory effects. It reportedly inhibits macrophage activation, neutrophil chemotaxis, and release of oxygen radicals and lysosomal enzymes. It influences coagulation by inhibiting platelet aggregation and potentially by inhibiting factor X activation. Alprostadil may promote fibrinolysis by stimulating production of tissue plasminogen activator. The overall benefits of iloprost and alprostadil are comparable, without significant differences in clinical efficacy or circulating markers of endothelial damage [100].

Epoprostenol, the first prostacyclin agent approved by the US Food and Drug Administration (FDA), in 1995, requires continuous intravenous infusion via a dedicated central venous catheter with infusion pump. Epoprostenol stimulates vasodilation of pulmonary and systemic arterial vascular beds and impedes platelet aggregation [11,101]. Based on published evidence, the initial dose of intravenous epoprostenol for treatment of refractory RP, with or without ischemic ulcers, should not exceed 2 ng/kg/min. A conservative titration schedule, based on those used in previous studies, should allow for rate increases of 1 ng/kg/min every 15 min as tolerated, adjusted as per the onset of treatment-emergent adverse effects. It should be noted that more aggressive uptitrations of 2 to 2.5 ng/kg/min were used in some studies. However, as a consequence of the lack of standardized efficacy outcomes in the available literature, it is not possible to assess if such regimens hold any advantages other than reaching the maximum dose more quickly. Intermittent infusions of 5 to 6 hours' duration should be initially considered to limit drug exposure and potential toxicities. However, it is reportedly reasonable to use continuous infusions for up to 72 h in patients unresponsive to intermittent therapy [101].

Epoprostenol is contraindicated in patients with congestive heart failure due to left ventricular dysfunction, and in those with known history of hypersensitivity reactions to the drug. Other adverse effects that should be monitored for include pulmonary edema, hemodynamic instability, line infections, and bleeding [101].

13. Endothelin Receptor Antagonists

Bosentan is an endothelin receptor antagonist (ERA) primarily used to manage severe pulmonary hypertension. A starting dose of 62.5 mg twice a day for four weeks, followed by 125 mg twice a day for 12 or 20 weeks, has shown some effectiveness in preventing formation of new digital ulcers, but did not influence healing of pre-existing ulcers [102,103]. Potential adverse effects include headaches, dizziness, and hypotension [16]. Adverse drug reactions are relatively mild, but during the treatment monthly liver function and 3-monthly full blood count is required [104,105].

14. Angiotensin II Receptor Blockers

Angiotensin II receptor blockers (ARBs) are reserved as a third-line treatment for mild Raynaud's phenomenon. There has been only one trial including 52 patients (25 patients with primary RP and 27 with SSc-related RP); this was an open-label, unblinded, controlled trial, during which a 12-week treatment with losartan 50 mg/day resulted in reduced frequency and severity of RP attacks in comparison to nifedipine 40 mg/day. The benefit was more noticeable in the subgroup of patients with primary RP. Losartan is widely available, accessible, and has an acceptable side effect profile [82,106,107].

15. Sulodexide

Sulodexide is a safe rheological drug used successfully as a supportive way of treating RP [84]. It consists of a purified mixture of glycosaminoglycans acquired from bovine intestinal mucosa, comprising a heparin of a rapidly moving field of electrophoresis (80%)

and dermatan sulfate (20%). It functions as an anticoagulant, is pro-fibrinolytic and anti-inflammatory, disrupts the process of fibrosis, and has a protective influence on vascular endothelial cells. Due to its pleiotropic activity and high safety profile, the benefits from sulodexide may be applied to many dermatological diseases [108].

SSc patients with secondary microcirculatory disorders who are intolerant to prostanoids, where there are contraindications, may be treated with sulodexide, 600 lipasemic units (LSU) intravenously twice a day. This dosing regimen has previously produced good therapeutic results. Other than sporadically observed dizziness and hypotension, no significant side effects were noted. In patients' pre-existing digital erosions and ulcers, a 3–4 day cycle of intravenous sulodexide, at 600 LSU twice a day every 4–6 weeks, has been shown to improve lesion healing [109].

Results of a recent pilot study suggest that the use of sulodexide treatment in RP results in a long-term improvement of capillary flow, a decrease in episode recurrence, and a reduction in pain intensity [110].

During parenteral treatment with sulodexide, it is imperative to discontinue any use of heparin or oral anticoagulants to reduce the bleeding risk [109].

16. Statins

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are extensively used to reduce serum cholesterol levels in the primary and secondary prevention of cardiovascular disease. Statins also have direct, vasculoprotective effects, which are independent of their ability to lower circulating LDL levels. Statins may be beneficial in RP patients, including those with SSc. Statins increase endothelial nitric oxide synthase expression and thus nitric oxide production, decrease oxidative stress, reduce endothelin-1 expression, impede endothelial apoptosis, increase endothelial progenitor cell mobilization, and promote microvascular growth [111,112]. Statins additionally inhibit endothelial–mesenchymal transition, which may contribute to vasculopathy and tissue fibrosis in SSc [113]. In a study of SSc patients, treatment with a statin was found to reduce the severity of RP vasospastic episodes and improved endothelial function. This was associated with increased levels of nitric oxide, reduced oxidative and inflammatory stress, increased quantity of endothelial progenitor cells, and amelioration of circulating concentrations of von Willebrand factor [114]. Data from a study performed by Abou-Raya et al. suggest that atorvastatin may exert beneficial effects in SSc by protecting the endothelium and improving its functional activity [115].

17. Topical Vasodilators

Topical vasodilators may be used as adjuvant therapy for RP patients. 10% nifedipine cream and 10% nitroglycerin gel have both been accepted as efficient therapeutic options with side-effects comparable to placebo usage. Local topical nitrates display significant efficacy in treatment of both primary and secondary RP [116]. Topical nitrates have been reported to increase perfusion at both distal digital ulcer and extensor digital ulcer cores in SSc patients, when compared to a placebo and evaluating with laser doppler imaging [117]. Wortsman et al. found that 5% sildenafil cream significantly improved blood flow in digital arteries (an increase of 9.2 mm/s, $p < 0.0083$). A trend toward improvement was also observed for vessel diameter in patients with SRP ($p = 0.0695$), suggesting local vasodilatation. Adverse effects to topical vasodilators include headaches and dizziness—no serious adverse effects were detected [118]. A study by Bentea et al. assessed the effects of nitroglycerin patch application to the dorsum of the hand. Results showed an increase in blood flow and hand temperature in patients with SSc after a cold challenge using laser doppler imaging [119].

18. Sympathectomy

Surgical treatment options involving sympathectomy or arterial reconstruction may be required in patients who suffer from incapacitating pain and ulcers with torpid evolution.

However, these techniques carry the risk of comorbidities and may not always provide satisfactory results.

Cautious selection of RP patients is necessary, and endoscopic thoracic sympathectomy should be reserved as an ultimate choice only for patients who have severe symptoms that are treatment-resistant with serious complications and impaired quality of life. The limiting factor with sympathectomy is a high recurrence rate. Symptoms and examination findings reported the quantity and dosage of medications used returned to preoperative levels in 66.6% of patients at month 6, and in all patients except one at the end of the 1st year [120].

Digital periarterial sympathectomy may be considered in patients suffering from critical digital ischemia or persistent ulceration despite aggressive vasodilatory therapy. A long-term retrospective study assessed 35 patients with primary or secondary RP who underwent thoracoscopic sympathectomy: 77% of participants had a positive response. However, symptoms recurred in 60% at a median follow-up of 5 months [121].

Single-port thoracoscopic sympathicotomy (SPTS) is a novel minimally invasive technique compared to conventional sympathectomy [122]. A recent study showed that the single-port procedure is effective in improving hand perfusion in patients with treatment-resistant RP. One month after unilateral single-port thoracoscopic sympathicotomy, the number of RP attacks was reduced and perfusion of the treated hand increased. However, the long-term efficacy and safety profile of this treatment need to be established [123].

19. Botulinum Toxin Type A

Botulinum toxin type A (BTX-A) is a polypeptide produced by the bacteria *Clostridium botulinum*. It is an acetylcholine release inhibitor in the peripheral nerve endings of the motor plate and sweat glands. It is well established that BTX-A inhibits acetylcholine release, leading to inhibition of neurotransmitter-induced vasoconstriction and relief of other symptoms, such as pain [124,125].

Botulinum toxins inhibit macromolecular SNARE complexes, which are involved in vesicle fusion with the plasma membrane, thus preventing neuronal exocytosis [126].

A study performed by Medina et al. established botulinum toxin as a safe, accessible, and effective therapeutic alternative for patients with severe RP, allowing those who do not respond to conventional treatments to sustain a good quality of life via annual infiltrations [127].

BTX-A is a promising non-surgical treatment modality and/or adjunct for patients who have contraindications to CCBs, PDE-5 inhibitors, and nitrates. It also provides a non-operative therapeutic alternative for patients experiencing chemotherapy-induced RP where mainstay therapies may be contraindicated, thus reducing pain, improving patient quality of life, and slowing disease progression [128].

In a study by Nagarajan et al. several patients derived long-term benefits from a single treatment, however in patients with SSc, repeat treatments were required and administered after an average of 6 months [129].

20. Riociguat

Riociguat is a first-in-class guanylate cyclase stimulator and may be a promising new treatment for RP. It works by direct stimulation of guanylate cyclase, independent from NO, and additionally via sensitization of guanylate cyclase to endogenous nitric oxide by stabilizing NO–guanylate cyclase binding. As a result, riociguat efficiently stimulates the nitric oxide–soluble guanylate cyclase–cyclic guanosine monophosphate pathway and leads to increased intracellular levels of cyclic guanosine monophosphate. In contrast to PDE-5 inhibitors, the action of riociguat does not depend on endogenous nitric oxide levels [130]. In the pilot study performed by Huntgeburth et al., a single oral dose of riociguat 2 mg was well tolerated in patients with RP and resulted in improved digital blood flow in some patient subsets, with high inter-individual variability [131].

21. SSRIs

An improvement in RP symptoms has been reported in patients treated with selective serotonin reuptake inhibitors (SSRIs). A study of 27 patients with SSc revealed that fluoxetine at a dose of 20mg/day was significantly superior to nifedipine in reducing the frequency and severity of RP attacks in patients with SSc [132]. Of note however, exacerbations of RP have also been reported with the use of serotonin reuptake inhibitors treatment [133].

22. Treat to Target (T2T) Strategy

At present, the decision to commence treatment and to evaluate response in RP—including the need for dose escalation—is principally based upon clinician–patient discussions regarding symptom severity, perceived effectiveness of the existing/planned interventions, and drug tolerability.

Hughes et al. proposed a five-stage roadmap that may support the development of a treat to target (T2T) strategy for SSc-RP. Significant initial steps are to define the study population and the goals of developing a T2T strategy (stage 1) and to review and shortlist candidate target items (stages 2 and 3, respectively). If agreement regarding feasible targets is not reached at this point, then the goals and purpose need to be refined. Subsequently, a consensus-building exercise among relevant stakeholders would allow the 'target' to be defined (stage 4). Ultimately, well-designed studies (stage 5) will be required to investigate the feasibility and treatment benefit of a T2T strategy in patients with SSc-RP. Much can be learned from primary studies of T2T for rheumatoid arthritis, including randomized trials comparing T2T with routine care, and those comparing different treatment approaches (e.g., monotherapy vs. combination therapy) to reach a defined target. Crucial features of these studies were the frequent review of patients, and the clear guidance that existed on how to intensify treatment of patients who had not reached the target [134,135].

23. Conclusions

An increased understanding of the pathogenesis of Raynaud's phenomenon is guiding new approaches to treatment. Assessment of peripheral endothelial dysfunction may aid identification of individuals with secondary Raynaud's phenomenon who are at risk of developing connective tissue diseases, and who may therefore benefit from repeated screening, early initiation of preventative treatments, and risk factor modification.

Capillaroscopy is of crucial value for the diagnosis and differentiation of primary and secondary Raynaud's phenomenon.

The presence of digital ulcers implies that intervention with vasodilator therapy is necessary. Early intervention is vital to the treatment of critical ischemia, and calcium channel blockers remain the first line of therapy. Alternatives for severe disease include phosphodiesterase-5 inhibitors and intravenous prostaglandin analogues. The overall benefits of iloprost and alprostadil are comparable without significant differences; however, ease of handling and the lower cost profile favours alprostadil. Sulodexide is a safe rheological drug successfully used as a supportive treatment in RP, resulting in a long-term improvement of capillary flow and a reduction in the frequency of Raynaud's syndrome relapse. Topical vasodilators, for example 10% nifedipine cream, 10% nitroglycerine gel, and 5% sildenafil cream, may act as adjuvant therapy. Riociguat may be a promising new treatment for Raynaud's phenomenon; however, this warrants further evaluation. A variety of alternative modalities have also been reported to be effective in the management of RP including botulinum toxin A, and sympathectomy, or single-port thoracoscopic sympathectomy.

Treat to target strategies may optimize treatment approaches for Raynaud's phenomenon and herald the emergence of disease-modifying vasodilator therapies for systemic sclerosis-related digital vasculopathy.

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ORIGINAL RESEARCH

Copeptin as a Biomarker of Microcirculation Alterations in Systemic Sclerosis

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Background: Systemic sclerosis is a connective tissue disease characterized by vasculopathy and progressive fibrosis, leading to multiorgan dysfunction. Given the complex and not fully elucidated pathogenesis, biomarkers of rapid disease progression and therapeutic response are lacking. Copeptin, which reflects vasopressin activity in serum, is used in diagnosing or prognosing different cardiometabolic conditions.

Objective: The aim of study was to investigate the concentration of copeptin in patients with systemic sclerosis and correlate it with specific clinical symptoms.

Patients and Methods: Serum copeptin was measured in patients with systemic sclerosis (34 women and 3 men; mean age 57.6 years) and in healthy individuals (n=30) using commercially available ELISA kits. According to the criteria of LeRoy our systemic sclerosis cohort consisted of 17 patients with limited cutaneous systemic sclerosis (45.9%) and 20 diffuse cutaneous systemic sclerosis patients (54.1%). According to the criteria of LeRoy our systemic sclerosis cohort consisted of 17 patients with limited cutaneous systemic sclerosis (45.9%) and 20 diffuse cutaneous systemic sclerosis patients (54.1%). The median duration of the disease was 10 [4–14] years.

Results: We found significantly higher copeptin concentration in patients with systemic sclerosis (4.21 pmol/L [3.04–5.42]) in comparison to control group (3.40 pmol/L [2.38–3.76], p<0.01). Copeptin significantly correlated with Raynaud's condition score (r=0.801, p<0.05). Patients with "late" capillaroscopic patterns had higher copeptin concentrations (5.37 pmol/L [4.29–8.06]) than patients with "early" (2.43 pmol/L [2.25–3.20], p<0.05) and "active" patterns (3.93 pmol/L [2.92–5.16], p<0.05]). Copeptin was found to be significantly higher in SSc patients with DUs (5.71 pmol/L [IQR 4.85–8.06]) when compared to SSc patients without DUs (3.31 pmol/L, [2.28–4.30], p<0.05). Additionally, copeptin concentration had good diagnostic accuracy in discriminating between patients with and without digital ulcers (AUC=0.863). Alprostadil decreased copeptin concentration from 4.96 [4.02–6.01] to 3.86 pmol/L [3.17–4.63] (p<0.01) after 4–6 cycles of administration.

Conclusion: Our findings suggest that copeptin may be a promising biomarker of microcirculation alterations in systemic sclerosis.

Keywords: serum biomarkers, vasculopathy, endothelial dysfunction, digital ulcers, systemic sclerosis

Introduction

Systemic sclerosis (SSc) is an autoimmune connective tissue disease associated with progressive fibrosis of the skin and internal organs, widespread peripheral vasculopathy, and immune dysfunction.[1] Although tissue fibrosis is the hallmark of SSc, vascular damage plays an important role in the early stages of disease pathogenesis and may precede fibrosis by months or years.[2] SSc-associated microvasculopathy clinically manifests as Raynaud's phenomenon, refractory ischemic digital ulcers (DUs), scleroderma renal crisis (SRC), or pulmonary arterial hypertension (PAH).[3–5] Digital ulcers occur in up to 50% of all SSc patients.[6] These disabling and painful lesions are recurrent, refractory to treatment and may lead to auto-amputation, infection, impaired hand function, and decreased quality of life.[7,8]

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Vascular alteration in SSc involves vasculopathy, thrombosis and deregulation of vascular tone control.[9] Blood vessel resistance is regulated by a complex interplay between sympathetic modulation, local and systemic vasoactive mediators, such as angiotensin II, endothelin-1 and vasopressin.[10] Angiotensin II (Ang II) is a highly active octapeptide involved in the regulation of blood pressure. Angiotensin II has two major receptor subtypes, type 1 (AT_1R) and type 2 (AT_2R), members of the seven transmembranes spanning the G protein-coupled receptor superfamily.[11] Although classically known for its role in the maintenance of circulatory system homeostasis, Ang II is now recognized as a mediator of inflammation and fibrosis through stimulation of AT_1R.[12,13] Pathophysiological significance of the disturbances in the angiotensin I/angiotensin II/angiotensin-(1-7) axis has been confirmed in SSc.[14] Endothelin-1 (ET-1) is another potent endogenous vasoconstrictor. Moreover, ET-1 causes fibrosis of the vascular cells, stimulates production of reactive oxygen species and proinflammatory cytokines.[15,16] Agonistic autoantibodies targeted against AT_1 and ET-1 type A receptors in patients with SSc predict mortality and are associated with more severe organ involvement.[17]

Vasopressin (AVP) is a vasoactive peptide that, like Ang II and ET-1, may be involved in vasoconstriction, inflammation, and fibrosis.[18] So far, AVP has been intensively studied in various pathologies of the cardiovascular system.[19] To the best of our knowledge, there were no studies of vasopressinergic system in SSc. However, the measurements of serum vasopressin concentrations are limited in clinical practice due to the short half-life time, low molecular stability and binding to platelets, which leads to a decrease in the actual peptide concentration.[20] Copeptin is the C-terminal part of pro-vasopressin, which is released together with AVP in equimolar amounts, making the copeptin an increasingly studied molecule to determine the vasopressinergic system activity. The role of copeptin as a biomarker has been examined in a variety of cardiovascular, kidney and metabolic disorders.[21–23]

Despite recent advances in SSc treatment, it is still challenging to identify patients at risk of rapid disease progression and development of severe organ complications. There is an urgent need to identify potential biomarkers of incipient vasculopathy before tissue damage becomes clinically apparent and irreversible.[24] The primary aim of this study was to assess copeptin concentration in patients with SSc in the context of its use as a biomarker reflecting the severity of SSc-related peripheral microvasculopathy.

Materials and Methods

Study Design

The study was cross-sectional and prospective in design. A cohort of consecutive outpatients and inpatients diagnosed with SSc from the tertiary referral dermatological and rheumatological centers in Warsaw were enrolled in this study from September 2019 to May 2022.

Patients

The study population consisted of adults with SSc, fulfilling the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) 2013 classification criteria[25] and age- and sex-matched healthy volunteers.

The exclusion criteria for patients and control group were as follows: heart failure (NYHA class III and IV or decompensated), unstable ischemic heart disease, history of acute cardiovascular disease event in the last 6 months, hypotension, uncontrolled arterial hypertension or diabetes mellitus, pulmonary hypertension, hepatic or renal failure, active malignancies or neoplastic disease whose treatment has been completed in the last 5 years (except for basal cell carcinoma), active or chronic infection, pregnancy and breastfeeding. Patients with SSc and overlapped autoimmune, rheumatic or connective tissue diseases were not enrolled in this study. Primary Raynaud's phenomenon was considered as an additional exclusion criterion for the control group.

Demographic, clinical, laboratory and immunological data were assessed according to polish and international expert recommendations.[26,27]

Clinical Assessment

All SSc patients underwent a thorough clinical examination with assessment of the following features: gender, age, body mass index (BMI), SSc subsets according to LeRoy et al[28] time of disease since the onset of Raynaud's phenomenon,

time of disease since the first non-Raynaud symptom, presence of digital ulcers, synovitis, joint contractures, muscle involvement, esophageal and gastrointestinal symptoms, dyspnea, arrhythmia, impaired exercise tolerance, comorbidities, smoking history, current and previous treatment.

The extent of skin involvement was evaluated by the modified Rodnan Skin Score (mRSS).[29] The skin involvement was assessed by only one assessor (M.S.) certified with Scleroderma Clinical Trials Consortium training.

Lung involvement was assessed via high-resolution computed tomography (HRCT) scan of the chest and pulmonary function tests (PFT), including forced vital capacity (FVC) and diffusing capacity for carbon monoxide (DLCO). Interstitial lung disease (ILD) was defined as the presence of pulmonary fibrosis on an HRCT (the study did not include a quantitative assessment of the lesions). Doppler echocardiography was used to screen for PAH. Gastrointestinal tract involvement was assessed with barium contrast X-ray of the esophagus and the stomach. These specialist diagnostic procedures were carried out in all patients with SSc as a part of regular follow-ups.

Laboratory Procedures

Laboratory parameters: whole blood count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) concentration, lipid profile, liver and kidney function tests, N-terminal pro-B-type natriuretic peptide (NT-proBNP) were quantified from peripheral blood during clinical routines. Antinuclear antibodies were evaluated by indirect immunofluorescence pattern on HEp-2 cells and detected by immunoblot analysis. Data on laboratory and immunological parameters were extracted from patients' medical records.

All blood samples were collected from the peripheral vein, between 6 a.m. and 7 a.m. (after at least 8h fasting period). Serum aliquots obtained by centrifugation (3000 rpm for 10 min at 4°C) were stored at −80°C until further analysis.

Serum copeptin concentrations were determined at baseline and after 4–6 cycles of alprostadil administration. Thirty-two patients received intravenous alprostadil (Prostavasin®, UCB Pharma GmbH, Monheim, Germany) for 3 consecutive days at a daily dose of 60 μg diluted in 50 mL of 0.9% sodium chloride administered as a 5-h infusion. Alprostadil was administered every 4–6 weeks for Raynaud phenomenon as well as for the prevention and treatment of digital ulcers. Copeptin was assessed using a commercial ELISA kit (Human Copeptin ELISA Kit, Bioassay Technology Laboratory, Shanghai, China), according to the manufacturer's instructions. All samples were tested in duplicate and performed in the same laboratory with the same instruments. The analytical sensitivity was 0.059 pmol/L. The coefficient of intra- and inter-assay repeatability was <8% and <10%, respectively.

Microvascular Assessment

The severity of Raynaud's phenomenon was assessed with the validated Raynaud's condition score (RCS).[30] It is completed every day by patients as an 11-point Likert scale and average scores are taken over a 1-week period. We analyzed mean nailfold video-capillaroscopy (NVC) was performed by the same investigator (MM) with USB digital microscope Dino-Lite CapillaryScope 200 MEDL4HMA (AnMo Electronics Corporation, Taiwan) in all SSc patients. Microvascular abnormalities were classified into early, active or late scleroderma pattern according to Cutolo et al.[31]

Ethical Approval

All patients participating in the study gave their written informed consent before entering the study. The study was conducted in accordance with the principles of the Helsinki Declaration and the protocol approved by the local ethical committee (KB/90/2018; Bioethics Committee at the Medical University of Warsaw, Warsaw, Poland).

Statistical Analysis

Statistical analysis was conducted with Statistica software v.13.3 (TIBCO, Palo Alto, CA, USA). Data were tested for normal distribution using the Shapiro–Wilk test. Descriptive statistics for continuous variables were reported as the mean and standard deviation (SD) or median and interquartile range (IQR), as appropriate. Categorical variables were expressed as a number and percentage. Due to non-normal data distribution, non-parametric tests were used as follows: Mann–Whitney U for two independent samples, Kruskal–Wallis test with post hoc Dunn analysis for multiple independent comparisons, Wilcoxon rank test for two paired samples. Correlations between continuous variables were evaluated

using the Spearman's rank-sum coefficient. Receiver operating characteristic (ROC) analysis was performed to define cut-off values. The p-values of <0.05 were statistically significant.

Results

Patients' Characteristics

Thirty-seven SSc patients (34 women and 3 men; mean age 57.6 years) and 30 age- and sex-matched control individuals (28 women, 2 men; mean age 53.7 years) were enrolled in this study. According to the criteria of LeRoy our SSc cohort consisted of 17 patients with limited cutaneous SSc (lcSSc; 45.9%) and 20 diffuse cutaneous SSc patients (dcSSc; 54.1%). The median duration of the disease was 10 years (IQR 4–14 years). Patients were treated with following vasoactive therapy: alprostadil (86.5%), calcium channel antagonists (21.6%), phosphodiesterase 5 inhibitors (51.3%), and sulodexide (67.6%). A summary of the main demographic, clinical, laboratory and serological characteristics of patients with SSc is shown in Table 1.

Table 1 The Characteristics of Patients with SSc and Control Group

	SSc Patients (n=37)	lcSSc (n=17)	dcSSc (n=20)	Control Group (n=30)
Age, years	57.6±11.6	63.6±8.2	52.4±11.8	53.7±12.1
Sex, n (%)				
Men	3 (8.1%)	1 (5.9%)	2 (10%)	2 (6.7%)
Women	34 (91.9%)	16 (94.1%)	18 (90%)	28 (93.3%)
BMI, kg/m^2	23.45 [21.89–26.78]	25 [22.89–29.07]	22.59 [21.09–25.27]	23.45 [21.89–26.78]
Modified Rodnan skin score	5 [3–9]	4 [2–6]	8 [5–15]	
Disease duration, years	10 [4–14]	9 [4–14]	11.5 [5.5–15.5]	
Autoantibody positivity, n (%)				
Antinuclear (ANA)	37 (100%)	17 (100%)	20 (100%)	
Anticentromere (ACA)	16 (43.2%)	16 (94.1%)	0 (0)	
Antitopoisomerase I (ATA)	17 (46.0%)	1 (5.9%)	16 (80%)	
Anti-RNA polymerase III	4 (10.8%)	0 (0)	4 (20%)	
Interstitial lung disease, n (%)	26 (70.3%)	9 (52.9%)	17 (85%)	
Esophageal dysmotility, n (%)	24 (64.9%)	10 (58.8%)	14 (70%)	
Raynaud's phenomenon, n (%)	37 (100%)	17 (100%)	20 (100%)	
Digital ulcers, n (%)	15 (40.5%)	5 (29.4%)	10 (50%)	
Nailfold capillaroscopy pattern, n (%)				
Early	6 (16.2%)	2 (11.8%)	4 (20%)	
Active	16 (43.2%)	9 (52.9%)	7 (35%)	
Late	15 (40.6%)	6 (35.3%)	9 (45%)	
Immunosuppressive therapy, n (%)				
Corticosteroids	0	0	0	
Cyclophosphamide	0	0	0	
Methotrexate	6 (16.2%)	2 (11.8%)	4 (20%)	
Mycophenolate mofetil	14 (37.8%)	6 (35.3%)	8 (40%)	
Vasoactive therapy, n (%)				
Alprostadil	32 (86.5%)	14 (82.3%)	18 (90%)	
Bosentan	0	0	0	
Calcium channel antagonist	8 (21.6%)	3 (17.6%)	5 (25%)	
Phosphodiesterase 5 inhibitors	19 (51.3%)	9 (52.9%)	10 (50%)	
Sulodexide	25 (67.6%)	15 (88.2%)	10 (50%)	

Abbreviations: SSc, systemic sclerosis; BMI, body mass index.

Copeptin in Systemic Sclerosis

In patients with SSc serum copeptin concentration was 4.21 pmol/L (IQR 3.04–5.42) and it was significantly higher than in control group (median 3.40 pmol/L, IQR 2.38–3.76, p<0.01, Figure 1).

Further, we examined the possible association of copeptin with different SSc clinical phenotypes. When SSc cutaneous subsets are concerned, patients with dcSSc (median 5.06 pmol/L, IQR 3.15–5.60) had higher serum copeptin concentration compared with lcSSc (median 4.02 pmol/L, IQR 2.96–4.71, p<0.05) and control group (p<0.01, Figure 1). There was no significant difference between lcSSc and control group. No significant correlation was found between copeptin concentration and age, BMI and disease duration.

Sixteen (43.2%) SSc patients had anticentromere (ACA) antibodies, 17 (46.0%) were positive for antitopoisomerase I (ATA) antibodies and 4 (10.8%) patients had anti-RNA polymerase III antibodies (anti-RNAP III). When patients were stratified according to autoantibodies, there was significant difference between anti-RNAP-patients (median 10.09 pmol/L, IQR 7.90–13.04) and other antibody types: ACA-patients (median 3.56 pmol/L, IQR 2.27–4.46, p<0.05) or ATA-patients (median 4.85 pmol/L, IQR 3.25–5.42, p<0.05, Figure 2).

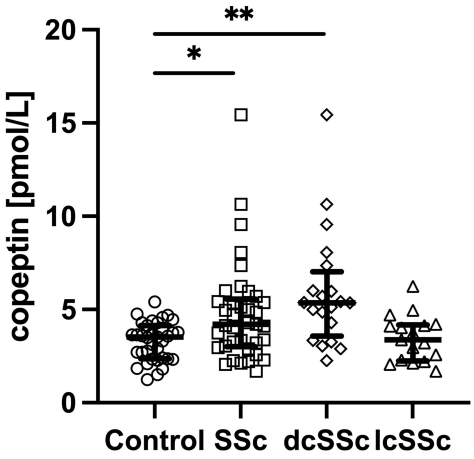

Figure 1 Serum concentration of copeptin in control group, patients with systemic sclerosis (SSc), diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc), *p<0.05, **p<0.01.

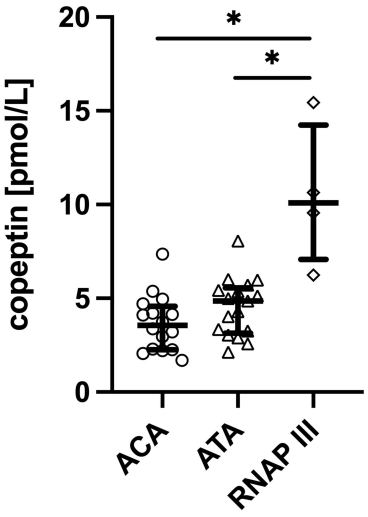

Figure 2 Serum concentration of copeptin in systemic sclerosis patients with anticentromere antibodies (ACA), antitopoisomerase antibodies (ATA) and anti-RNA polymerase III antibodies (RNAP III). *p<0.05.

When analyzing copeptin concentration in SSc patients according to the different organ manifestations, no differences were found regarding the extent of skin fibrosis measured by mRSS, prevalence of interstitial lung disease (ILD) as well as patients with esophageal dysmotility.

Copeptin and Microvascular Impairment in SSc

All patients reported the presence of Raynaud's phenomenon. We found a significant correlation between serum copeptin concentration and Raynaud's condition score (rho = 0.801, $p<0.05$, Figure 3).

The NVC pattern was found "early" in 6 (16.2%), "active" in 16 (43.2%) and "late" in 15 (40.6%) patients. When comparing copeptin concentration in SSc patients with different NVC patterns, the values of copeptin were significantly higher in the "late" pattern (median 5.37 pmol/L, IQR 4.29–8.06) in comparison to "early" (median 2.43 pmol/L, IQR 2.25–3.20, $p<0.05$) and "active" patterns (median 3.93 pmol/L, IQR 2.92–5.16, $p<0.05$, Figure 4).

In patients receiving alprostadil, serum concentration of copeptin significantly decreased from 4.96 pmol/L (IQR 4.02–6.01) to 3.86 pmol/L (IQR 3.17–4.63, $p<0.01$) after 4–6 cycles of drug administration (Figure 5).

As far as digital ulcers are concerned, copeptin was found to be significantly higher in SSc patients with DUs (median 5.71 pmol/L, IQR 4.85–8.06) when compared to SSc patients without DUs (median 3.31 pmol/L, IQR 2.28–4.30, $p<0.05$) and control group ($p<0.05$, Figure 6).

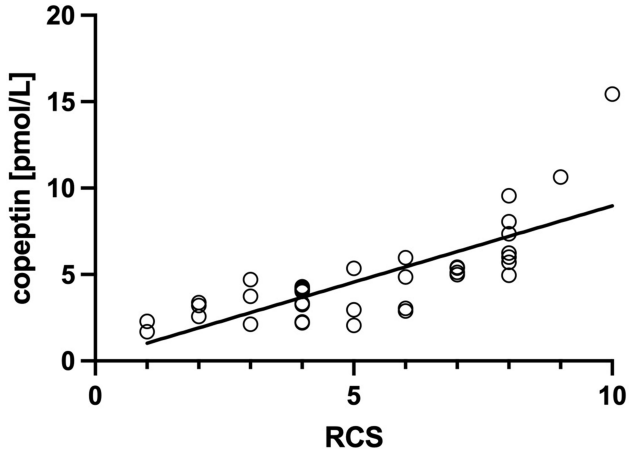

Figure 3 Correlation of copeptin with Raynaud's condition score (RCS; r=0.801, $p<0.05$).

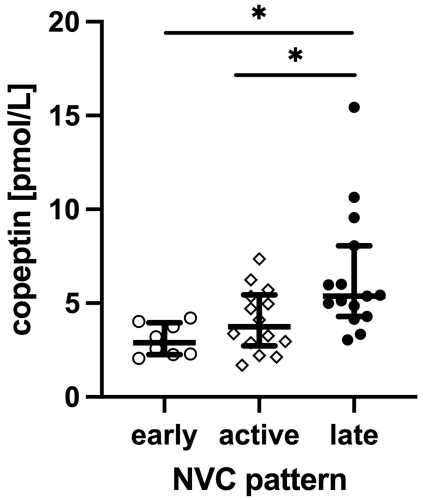

Figure 4 Serum concentration of copeptin in systemic sclerosis patients with "early", "active" and "late" nailfold video-capillaroscopy (NVC) patterns, *$p<0.05$.

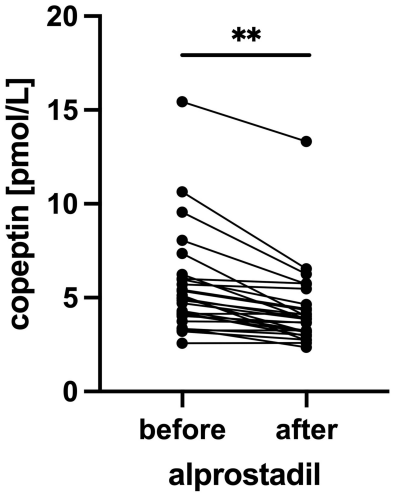

Figure 5 Serum concentration of copeptin in systemic sclerosis patients before and after 4–6 cycles of intravenous alprostadil administration. **p<0.01.

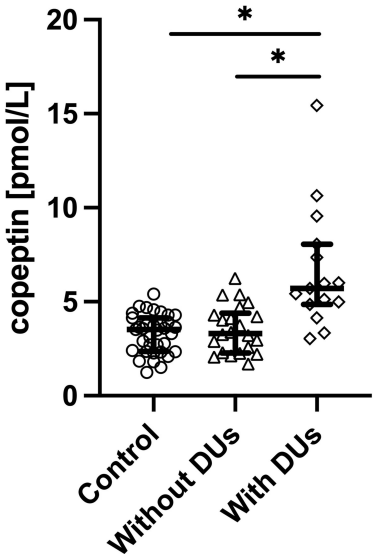

Figure 6 Serum concentration of copeptin in control group, patients with systemic sclerosis without and with digital ulcers (DUs). *p<0.05.

The ROC curve analysis revealed copeptin cut-off value of 4.86 pmol/L in discriminating between SSc patients with and without DUs (sensitivity 80.0%, specificity 81.0%, area under the curve [AUC] = 0.863, Youden's index 0.61, Figure 7).

Copeptin and Immunosuppression

Twenty patients with SSc (54.1%) received immunosuppressive therapy, while 17 (45.9%) were without immunosuppression. Among patients treated with immunosuppressive agents, 12 (60%) have dcSSc, 8 (40%) ACA, 8 (40%) ATA, 4 (20%) anti-RNAP III antibodies, 3 (15%) early, 8 (40%) active and 9 (45%) late NVC pattern. Respectively, in patients without immunosuppression, 8 (47.1%) have dcSSc, 9 (52.9%) ACA, 8 (47.1%) ATA, 0 anti-RNAP III antibodies, 3 (17.6%) early, 8 (47.1%) active and 6 (35.3%) late NVC pattern. There were no changes in copeptin concentration between patients currently treated with immunosuppressive drugs (median 4.25 pmol/L, IQR 3.56–5.40) and patients without immunosuppressive treatment (median 3.25 pmol/L, IQR 2.58–5.71, p=0.437).

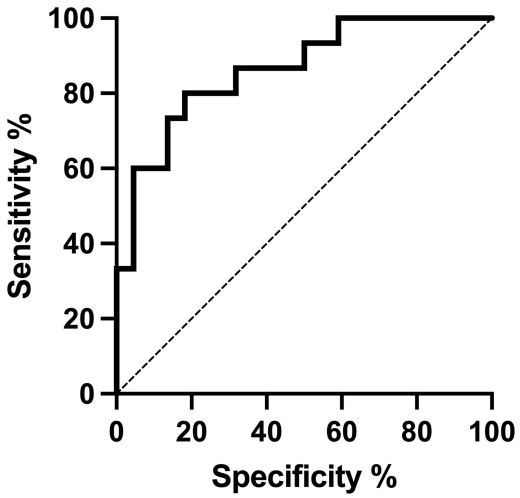

Figure 7 ROC curve analysis of copeptin in SSc patients without digital ulcers (DUs) vs SSc patients with DUs.

Discussion

The key finding of this study is that increased copeptin concentration in patients with SSc is significantly associated with microcirculation dysfunction which presents as Raynaud's phenomenon severity, nailfold capillary abnormalities and the presence of digital ulcers. Determination of serum copeptin concentration reflects changes in the activity of vasopressinergic system.[32] Vasopressin is known to play an essential role in microcirculation regulation through activation of three receptor subtypes (V1a, V1b and V2). V1a receptors are expressed on the vascular smooth muscle cells and promote vasoconstriction. Activation of V1b receptors is part of the adaptive response to stress leading to stimulation of the hypothalamic-pituitary-adrenal axis. V2 receptors are associated with water reabsorption in the kidneys and release of coagulation factors from endothelial cells, such as von Willebrand factor and coagulation factor VIII.[33,34]

We found a significant correlation between serum copeptin concentration and Raynaud's condition score, which is a validated outcome measure. Raynaud's phenomenon (RP) is caused by an imbalance between endogenous vasoconstrictive agents (endothelin-1, angiotensin II) and endothelium-derived vasodilators (nitric oxide, prostacyclin).[35] Vasopressin is another potent vasoconstrictor that is hypothesized to contribute to excessive vasoconstriction in RP.[36,37]

Moreover, relcovaptan (SR-49059), selective V1a receptor antagonist, has shown initial positive results in the treatment of Raynaud's disease.[37] Our study is the first evaluating the copeptin/vasopressin system in SSc-related microcirculation alterations. However, the diagnostic and prognostic value of copeptin has been reported also in other systemic diseases characterized by microangiopathy. Several studies indicated that AVP contributes to the development and progression of diabetic microangiopathy and serum copeptin may serve as an independent marker of renal, heart or retinal microcirculation involvement in type 2 diabetes patients.[38]

In our SSc group, higher concentrations of serum copeptin were observed in patients presenting "late" NVC pattern, which is characterized by irregular enlargement of the capillaries, few or absent giant capillaries and haemorrhages, ramified and bushy capillaries and progressive capillary loss (50–70%) with formation of extensive avascular areas (vascular desertification). These alterations, namely abnormal vessel morphology, changes in capillary number and disorganization of microcirculation architecture observed in the "late" NVC pattern stem from insufficient angio- and vasculogenesis.[39,40] Inadequate processes of new blood vessels formation do not allow the regeneration of functional microcirculation and are related to imbalance between pro-angiogenic and angiostatic factors.[41] Increased concentration of copeptin in SSc patients presenting "late" NVC pattern suggests that AVP might contribute to insufficient angiogenic response. This hypothesis is in accordance with the results from preclinical breast and colorectal cancer models showing that desmopressin, vasopressin peptide analog and a strong agonist of V2 receptor, reduces tumor-induced angiogenesis by production of endogenous angiostatic molecules (angiostatin) and reduction of the expression of different promoters of endothelial growth.[42,43] Impaired neovascularization in SSc contributes to the development of DUs.[44] In our cohort of

SSc patients increased copeptin concentration was related to the presence of ischemic DUs. This finding confirms the importance of vasopressin in peripheral microcirculation disorders in the course of SSc and suggests that monitoring of copeptin concentrations may help to predict the prognosis of microangiopathy. While copeptin has not been studied in SSc so far, its properties as a biomarker of microcirculation abnormalities were studied in diabetic angiopathy.[38] Baseline serum copeptin is associated with cumulative incidence of lower-extremity amputations in cohorts of patients with type 1 and type 2 diabetes and may help to identify high-risk individuals.[45] Further studies are needed to confirm prospectively that copeptin may serve as a diagnostic tool for discriminating increased risk of DUs development. Noteworthy, we document that in SSc patients receiving alprostadil (prostaglandin E1 analog), serum concentration of copeptin significantly decreased after 4–6 cycles of drug administration. Intravenous prostaglandin analogs, such as alprostadil and iloprost (prostaglandin I2 analog) have been reported to be effective in RP secondary to SSc as well as improve DUs healing and prevent DUs in patients with history of DUs.[46,47] The known effects of alprostadil include inhibition of vasoconstrictive effects of noradrenaline, Ang II, ET-1 and vasopressin.[48] Additionally, PGE may modulate vasopressin release and antagonizes AVP at intracellular signaling pathway level.[49,50]

To the best of our knowledge, this is the first study evaluating the serum concentration of copeptin in patients with systemic sclerosis. We observed increased copeptin concentration in dcSSc when compared to the lcSSc and control group. Diffuse cutaneous SSc is the subtype characterized by rapid progression and a high prevalence of early internal-organ involvement with interstitial lung disease and cardiac involvement (including pulmonary arterial hypertension) being the main causes of SSc-associated mortality.[1] No differences between the control and lcSSc, despite the fact that microcirculation disorders are an important component of this subtype, may result from the applied rheological treatment or the small study group. Autoantibody profiles in SSc are predictive of disease course and organ involvement.[51] When patients were stratified according to autoantibodies there was a significant increase in copeptin concentration in anti-RNAP III-positive patients. This is in accordance with several observations showing that SSc patients with positive anti-RNAP III antibodies had more frequently rapidly progressive diffuse skin thickening and higher frequency of vascular involvement, such as scleroderma renal crisis, pulmonary hypertension and ischemic damage associated with RP.[52] We did not find significant differences in copeptin concentration in SSc patients regarding the age, BMI, extent of skin fibrosis, prevalence of ILD and esophageal dysmotility. The possible reason for this could be that the number of participants was small. However, strict entry criteria limiting the number of study participants with severe organ damage which, independently of SSc, are associated with elevated copeptin concentration were obligatory to reduce possible factors that might influence baseline copeptin.

Based on our analysis, it may be suggested that evaluation of serum copeptin is useful for identifying SSc patients who are at risk of vascular complications. Thus, it may be hypothesized that determining the copeptin level may help individualize the management of SSc by intensification of rheological treatment in patients with high-risk of certain complications such as progression of NVC abnormalities or the DU development. There may be some possible limitations such as cross-sectional and single-center type of study as well as a relatively small sample size. However, these data may pave the way for subsequent prospective investigations which clarify copeptin significance as a biomarker in SSc.

Conclusions

Vascular involvement plays an essential role in SSc pathogenesis. The unmet need in translational investigations is to obtain biomarkers which will be useful in disease prevention, prognosis and treatment. We demonstrate that increased copeptin concentration in SSc is associated with more severe peripheral microcirculation impairment, such as Raynaud's phenomenon, NVC changes and digital ulcers. Copeptin may represent a promising potential biomarker in SSc, which allows for personalized therapy. Further prospective studies on larger cohorts of patients with evaluation of how different vascular therapies impact copeptin concentration are needed before its measurements may be used in clinical practice.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of Medical University of Warsaw (KB/90/2018).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

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Disclosure

The authors declare no conflict of interest.

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ORIGINAL RESEARCH

Hypoxia-Inducible Factor-1α (HIF-1α) as a Biomarker for Changes in Microcirculation in Individuals with Systemic Sclerosis

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ABSTRACT

Introduction: Systemic sclerosis is an autoimmune disease characterized by tissue fibrosis and microangiopathy. Vascular changes such as a decrease in capillary density diminish blood flow and impair tissue oxygenation. Reliable ways to monitor disease activity and predict disease progression are desired in the process of patient selection for clinical trials and to optimize individual patient outcomes. Hypoxia-inducible factor-1 (HIF-1) is a dimeric protein complex that plays an integral role in the body's response to hypoxia. Our study aimed to investigate the potential abnormalities of HIF-1α plasma concentration and its possible association with disease activity and vascular abnormalities in patients with systemic sclerosis.

Methods: Blood plasma levels of HIF-1α were measured in patients with systemic sclerosis ($n = 50$) and in healthy individuals ($n = 30$) using commercially available ELISA test kits.

Results: The results showed a marked increase in HIF-1α levels in patients with systemic sclerosis (3.042 ng/ml [2.295–7.749]) compared to the control group (1.969 ng/ml [1.531–2.903] $p < 0.01$). Patients with diffuse cutaneous SSc (2.803 ng/ml, IQR 2.221–8.799) and limited cutaneous SSc (3.231 ng/ml, IQR 2.566–5.502) exhibited elevated serum HIF-1α levels compared to the control group ($p < 0.01$). We found a notable increase in HIF-1α plasma concentration in patients with an "active" pattern (6.625 ng/ml, IQR 2.488–11.480) compared to those with either an "early" pattern (2.739, IQR 2.165–3.282, $p < 0.05$) or a "late" pattern (2.983 ng/ml, IQR 2.229–3.386, $p < 0.05$). Patients with no history of digital ulcers had significantly higher levels of HIF-1α (4.367 ng/ml, IQR 2.488–9.462) compared to patients with either active digital ulcers (2.832 ng/ml, IQR 2.630–3.094, $p < 0.05$) or healed digital ulcers (2.668 ng/ml, IQR 2.074–2.983, $p < 0.05$).

Conclusions: Our results indicate that HIF-1α may serve as a biomarker in assessing microcirculatory changes in individuals with systemic sclerosis.

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Keywords: Biomarker; Digital ulcers; HIF-1α; Hypoxia-inducible factor-1α; Microangiopathy; Systemic sclerosis

Key Summary Points

Why carry out this study?

The study investigated the potential use of hypoxia-inducible factor-1α (HIF-1α) as a biomarker in assessing microcirculatory changes in individuals with systemic sclerosis (SSc). There is an unmet need to identify reliable ways to monitor disease activity and predict disease progression to optimize individual patient outcomes and to select patients for clinical trials

The study explored the association between HIF-1α plasma concentration and disease activity and vascular abnormalities in patients with SSc

What was learned from the study?

HIF-1α plasma concentration was significantly increased in patients with SSc compared to healthy individuals, irrespective of disease subtype

The study suggests a potential role of HIF-1α as a valuable biomarker for assessing microcirculatory changes

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disease defined by vascular and immune dysfunction, manifesting primarily as fibrosis of the skin and internal organs [1]. Two main subsets of SSc are described according to the extent of skin involvement: limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc) [2]. Microangiopathy and Raynaud's phenomenon are the clinical hallmarks and can be found in almost all patients from the earliest stages of the disease [3, 4]. The gradual reduction in the quantity and diameter of vessels afflicted by SSc leads to chronic hypoxia in both the skin and organs of affected individuals [5]. The vascular aberrations observed in SSc are marked by capillary loss and structural alterations. Alterations in the capillary network can be seen early in the course of SSc using nailfold capillaroscopy (NFC) [6]. Proximal nailfold capillary abnormalities have been associated with the development of digital ulcers (DU) [7]. Ulcers on the fingertips pose a significant clinical obstacle because of the accompanying pain, risk of infection, and eventual necrosis [8, 9].

During the last 15 years, attempts have been directed toward identifying SSc at an early stage. Previously suggested criteria, known as VEDOSS, aim to detect early signs and symptoms of SSc in patients with Raynaud's phenomenon [10]. Patients with all signs or symptoms of the VEDOSS criteria already fulfill the 2013 American College of Rheumatology–European League Against Rheumatism (ACR–EULAR) classification criteria for SSc [11]. Patients who satisfy the criteria for early diagnosis of SSc often already exhibit digital ulcers [10].

Inflammation and microvascular dysfunction appear to be the primary events that progressively trigger the fibrotic process [12, 13]. The precise etiology of fibrotic changes remains only partially understood but may include impaired communication between endothelial cells, epithelial cells, and fibroblasts, as well as lymphocyte activation, autoantibody production, inflammation, and tissue fibrosis [14]. Angiogenesis, the formation of newly formed capillaries from preexisting vessels via a well-programmed cascade of events, is dysregulated in SSc and cannot ensure an efficient vascular recovery. Vascular injury induces hypoxia and tissual ischemia which are the primary triggers for angiogenesis [15].

Markers of endogenous hypoxia as well as molecular responses to hypoxia have been thoroughly detailed over the past two decades.

A growing body of evidence indicates that hypoxia-inducible factor-1α (HIF-1α), a key transcriptional factor involved in the response to chronic hypoxia, may be implicated in the pathogenesis of fibrotic diseases such as SSc [16]. HIF-1 is a transcription factor that

responds to changes in oxygen levels and enables organisms to adapt to low-oxygen environments. HIF-1 is composed of two different subunits: one α-subunit that is regulated by oxygen levels, and one β-subunit that is expressed continuously regardless of oxygen levels [17]. Each subunit of HIF-1 contains basic helix-loop-helix-PAS (bHLH-PAS) domains that facilitate the binding of the two subunits together and to DNA hypoxia response elements (HREs) [18, 19]. Since there excess HIF-1β is present in vivo, the transcriptional activity of HIF-1 is mainly determined by the levels of the HIF-1α subunit [20]. In addition to HIF-1, there are two other isotypes in the hypoxia-inducible factor family, known as HIF-2 and HIF-3, that also play a role in transcriptional responses to hypoxia, immunity, neovascularization, and other stimulators [21, 22]. However, HIF-1 is considered one of the most important hypoxia-inducible factors involved in cellular metabolism, tissue repair, and inflammation [22, 23]. The suspected functions of HIF-1 include stimulation of excessive extracellular matrix, vascular remodeling, and futile angiogenesis with further exacerbation of chronic hypoxia [22]. HIF-1α signaling is associated with cardiovascular, inflammatory, infectious, and metabolic diseases [24, 25]. The rs12434438 polymorphism of the HIF-1 gene has been linked with a predisposition to developing SSc [26].

Takagi et al. [27] demonstrated that the *HIF1A* gene is a risk factor for developing pulmonary arterial hypertension (PAH) in patients with SSc. The authors found that the AA genotype at rs12434438 was associated with a subset of patients with SSc and severe PAH, suggesting that the rs12434438 single nucleotide polymorphism (SNP) may contribute to the development of PAH with SSc [27].

Results of the study conducted by Mao et al. [28] suggest that the HIF-1α/vascular endothelial growth factor (VEGF) signaling pathway may have a critical role in mediating the hypoxia-induced endothelial to mesenchymal transition (EndMT) seen in the cutaneous microcirculation of patients with SSc [28]. It has been suggested that endothelial cell damage is a key event that triggers vascular remodeling, intimal arteriole growth, capillary collapse, and ultimately blood vessel occlusion [29]. The importance of EndMT in the pathophysiology of tissue fibrosis and fibroproliferative vasculopathy, observed in various fibrotic diseases, has been firmly established [30]. Thus, the role endothelial cells (ECs) play in the vascular alterations associated with SSc, as well as the identification of associated biomarkers, are common subjects of ongoing research [31, 32].

A remarkable breakthrough regarding early diagnosis of the disease was made a few years prior with the introduction of the new guidelines enabling the diagnosis of SSc before the onset of overt fibrotic symptoms [11]. Appropriate early augmentation of treatment can prevent pathological vascular remodeling and therefore abate the process of fibrosis [33, 34]. With the advent of advanced immunodiagnostic techniques, many autoantibodies specific to SSc have been described. Many of these autoantibodies help predict clinical manifestations such as internal organ dysfunction and the extent of skin involvement [35]. However, identifying patients who are at risk of developing digital ulcers and determining which patients are responding to vasoactive therapy remains a challenge. For these reasons, the aim of our study was to investigate the differences between plasma concentration of HIF-1α, disease activity, and vascular abnormalities in patients with SSc [36, 37].

METHODS

The study was cross-sectional and prospective in design.

Patients

A total of 50 patients who were diagnosed with SSc in accordance with the 2013 ACR/EULAR classification criteria [11] were recruited.

Exclusion criteria included respiratory diseases (PAH combined with interstitial lung disease, asthma, tuberculosis, pneumonia, bronchial pneumonia, lung cancer, and other pulmonary organic diseases), cardiovascular diseases (history of heart failure or cardiomyopathy; coronary heart disease); chronic kidney

disease (stage 3b–5); liver fibrosis; hemolytic disease; active neoplastic disease or neoplastic disease whose treatment has been completed in the last 5 years (except for basal cell carcinoma), pancytopenia or anemia, pregnancy, breast-feeding, and acute infection or inflammation.

The control group consisted of individuals matched for age, sex, and body mass index (BMI). The presence of primary Raynaud's phenomenon was considered an additional exclusion criterion in the control group.

All study participants underwent a comprehensive physical examination, and their demographic data was collected via a questionnaire. A detailed medical history was recorded, establishing the absence or presence of pulmonary disorders such as pulmonary hypertension and/or pulmonary fibrosis, esophageal motility disorders (which were documented through imaging or endoscopic examinations), and the duration of the disease. In addition, medication use and cardiovascular risk factors (such as smoking and hypertension) were also documented.

Clinical Assessment

The clinical assessments included quantification of various laboratory parameters such as complete blood count, erythrocyte sedimentation rate (ESR), concentration of C-reactive protein (CRP), lipid profile, as well as liver and kidney function tests. Additionally, the level of N-terminal pro-B-type natriuretic peptide (NT-proBNP) was also measured from the peripheral blood during routine checkups. Indirect immunofluorescence and immunoblot analysis were performed on HEp-2 cells to evaluate the presence of antinuclear antibodies. Laboratory and immunological parameters were extracted from the patient's medical records.

Microvasculature Assessment

The same investigator (MM) conducted a nailfold video-capillaroscopy (NVC) examination on all patients with SSc using a Dino-Lite CapillaryScope 200 MEDL4HMA USB digital microscope (AnMo Electronics Corporation, Taiwan).

The capillaroscopic examination involved acclimatizing the patients to room temperature for 15–20 min (approximately 25 °C), followed by examining eight fingers (II to V bilaterally). To aid in imaging, a drop of paraffin oil was applied to the nailfold area, and four images were captured for each examined finger.

The microvascular abnormalities observed were categorized as either early, active, or late scleroderma patterns based on the criteria set forth by Cutolo et al. [10].

Measurement of Serum HIF-1α Concentration

Blood samples were collected once after an overnight fast. The blood samples were then centrifuged at 4000 rpm ($1500\times g$) for 10 min within 15 min of their collection. The plasma was subsequently collected and frozen at − 80 °C to be analyzed later.

HIF-1α concentration was evaluated using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Human HIF-1A ELISA Kit, Bioassay Technology Laboratory, Shanghai, China), following the manufacturer's instructions. Each sample was subjected to duplicate testing using identical equipment and procedures in a single laboratory. The analytical sensitivity was 0.01 ng/ml. For the ELISA analyses mentioned above, intra-assay coefficients of variation were below 4.9% and inter-assay coefficients of variation were below 10%.

Statistical Analysis

Statistical analysis was performed using Statistica software v.13.3 (TIBCO, Palo Alto, CA, USA). The normal distribution of data was checked using the Shapiro–Wilk test. Descriptive statistics for continuous variables were presented as either mean and standard deviation (SD) or median and interquartile range (IQR), depending on the distribution of data. Categorical variables were expressed as numbers and percentages. Non-parametric tests were used because of the non-normal distribution of data. The Mann–Whitney U test was used for two independent samples, the Kruskal–Wallis

test with post hoc Dunn analysis was used for multiple independent comparisons, and the Wilcoxon rank test was used for two paired samples. Correlations between continuous variables were analyzed using Spearman's rank-sum coefficient. Statistical significance was considered at p values less than 0.05.

Ethical Statement

All participants gave their written informed consent before entering the study. The study was performed in accordance with the Helsinki Declaration and the ethics committee's authorized protocol (KB/90/2018 of 21 May 2018; Bioethics Committee at the Medical University of Warsaw, Warsaw, Poland).

Between September 2019 and May 2022, a cohort of individuals who had been diagnosed with SSc were recruited and subsequently attended outpatient or inpatient services.

RESULTS

Patient Characteristics

Table 1 depicts the anthropometric characteristics of the study population. A total of 50 Caucasian patients with SSc (42 women, 8 men), with a mean age of 56.4 years, along with 30 control individuals matched in age and sex (25 women, 5 men; mean age 52.1 years), were included in the study.

HIF-1α Plasma Concentration in Systemic Sclerosis

The HIF-1α plasma concentration in patients with SSc was 3.042 ng/ml (IQR 2.295–7.749), which was significantly higher compared to the control group (median 1.969 ng/ml, IQR 1.531–2.903, $p < 0.01$; Fig. 1).

In addition, we investigated whether there was a difference between HIF-1α plasma concentration and different clinical phenotypes of SSc. With regards to cutaneous subsets of SSc, patients with dcSSc (2.803 ng/ml, IQR 2.221–8.799) and those with lcSSc (3.231 ng/ml, IQR 2.566–5.502) had a higher HIF-1α plasma concentration compared to the control group ($p < 0.01$). However, there was no significant difference in HIF-1α plasma concentration between the lcSSc and the dcSSc group (Fig. 1).

We did not observe any significant differences between HIF-1α plasma concentration and age, BMI, Raynaud's phenomenon, or disease duration.

When the patients were grouped according to autoantibody production, no significant difference was observed between patients positive for anti-RNA polymerase III (anti-RNAP) and patients with other antibody types such as anticentromere autoantibodies (ACA) or anti-topoisomerase I autoantibodies (ATA). After analyzing the HIF-1α plasma concentration in patients with SSc, there were no observable differences in the extent of skin fibrosis when assessed by the modified Rodnan skin score (mRSS) and pulmonary hypertension or esophageal dysmotility. We did not observe differences in serum HIF-1α concentration between patients with and those without interstitial lung disease nor did we find a correlation between HIF-1α concentration and the carbon monoxide diffusion capacity (DLCO).

Association Between HIF-1α Concentration and Microvascular Dysfunction in Systemic Sclerosis

After comparing HIF-1α plasma concentration in patients with SSc and different nailfold video-capillaroscopy patterns, we observed a significant increase in HIF-1α plasma concentrations in patients with the "active" pattern of the disease (6.625 ng/ml, IQR 2.488–11.480) compared to those with the "early" pattern (2.739, IQR 2.165–3.282, $p < 0.05$) and the "late" pattern (2.983 ng/ml, IQR 2.229–3.386, $p < 0.05$, Fig. 2).

Regarding digital ulcers in the last 12 months, in patients with SSc, those without DUs had significantly higher plasma concentrations of HIF-1α (4.367 ng/ml, IQR 2.488–9.462) compared to patients with either active DUs (2.832 ng/ml, IQR 2.630–3.094,

Table 1 Characteristics of the individuals with systemic sclerosis and control group

	Systemic sclerosis (*n* = 50)	**Control (*n* = 30)**	***p* value**
General characteristics			
Age, years, mean (SD)	56.4 (11.8)	52.1 (11.7)	0.12
Sex, women, *n* (%)	42 (84.00%)	25 (83.33%)	0.94
Body mass index, kg/m^2	23.56 (21.31–27.44)	25.29 (22.35–26.25)	0.67
Disease subset, *n* (%)			
Limited cutaneous systemic sclerosis	27 (54%)	–	–
Diffuse cutaneous systemic sclerosis	23 (46%)	–	–
Systemic sclerosis duration since the diagnosis, years	6 (4–13)	–	–
Autoantibody positivity, *n* (%)			
Antinuclear (ANA)	50 (100%)	–	–
Anticentromere (ACA), *n* (%)	23 (46%)		–
Antitopoisomerase I (Topo I), *n* (%)	19 (38%)	–	–
Anti-RNA polymerase III, *n* (%)	5 (10%)	–	–
Interstitial lung disease, *n* (%)	29 (58%)	–	–
Esophageal dysmotility, *n* (%)	27 (54%)	–	–
Modified Rodnan skin score	4 (IQR 2–9)	–	–
Raynaud's phenomenon, *n* (%)	50 (100%)	–	–
Digital ulcers, *n* (%)	18 (36%)	–	–
Nailfold capillaroscopy pattern, *n* (%)			
"Early"	12 (24%)	–	–
"Active"	24 (48%)	–	–
"Late"	14 (28%)	–	–
Immunosuppressive therapy, *n* (%)			
Cyclophosphamide	3 (6%)	–	–
Methotrexate	12 (24%)	–	–
Mycophenolate mofetil	14 (28%)	–	–
Vasoactive therapy, *n* (%)			
Alprostadil	45 (90%)	–	–
Bosentan	0 (0%)	–	–
Calcium channel antagonist	9 (18%)	–	–
Phosphodiesterase 5 inhibitors	27 (54%)	–	–
Sulodexide	29 (58%)	–	–

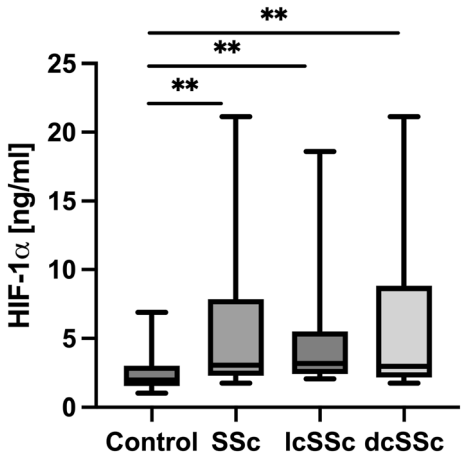

Fig. 1 Comparison of the hypoxia-inducible factor-1α (HIF-1α) plasma concentration [ng/ml] in control group, all individuals with systemic sclerosis (SSc), patients with limited cutaneous SSc (lcSSc), and patients suffering from diffuse cutaneous SSc (dcSSc)

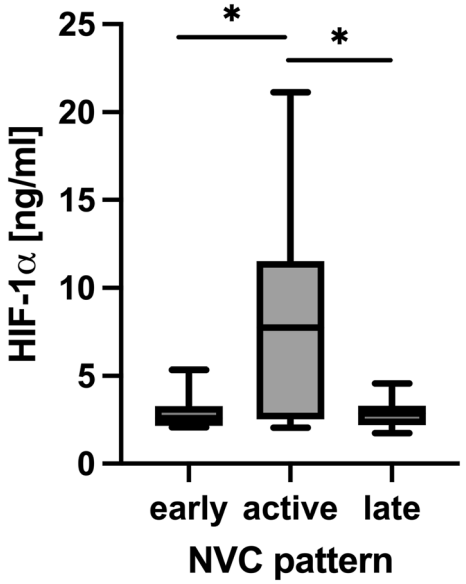

Fig. 2 Comparison of the hypoxia-inducible factor-1α (HIF-1α) plasma concentration [ng/ml] in patients with systemic sclerosis (SSc) and different nailfold capillaroscopy (NFC) patterns: "early", "active", and "late"

$p < 0.05$) or healed DUs (2.668 ng/ml, IQR 2.074–2.983, $p < 0.05$) (Fig. 3).

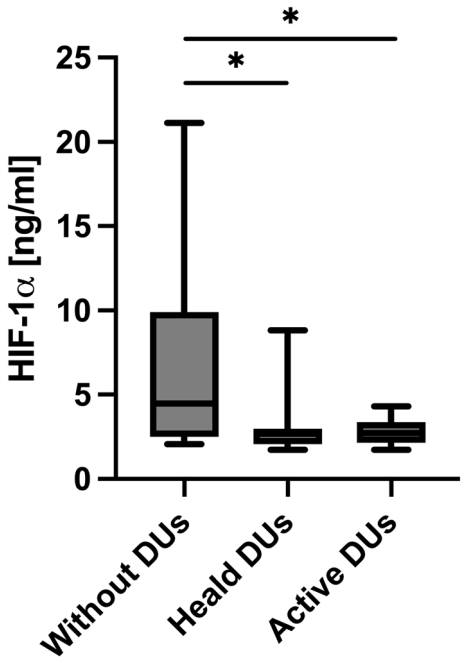

Fig. 3 Comparison of the hypoxia-inducible factor-1α (HIF-1α) plasma concentration [ng/ml] in patients with systemic sclerosis without digital ulcers (DUs), with healed DUs, and with active DUs

DISCUSSION

We observed a significant elevation in the HIF-1α plasma concentration among patients with SSc compared to the control group. This finding held true for both the subset of patients with diffuse cutaneous systemic sclerosis and limited cutaneous systemic sclerosis.

The findings of our research are consistent with those reported by Heger et al. [38], which showed that patients with SSc and secondary Raynaud's syndrome have higher serum levels of HIF-1α protein and messenger ribonucleic acid (mRNA) expression in monocytes than healthy control subjects. Heger's study included patients with both diffuse and limited SS subtypes, with approximately 40% of them having digital ulcers at the time of inclusion [38]. Mao et al. [28] reported increased expression of HIF-1α in skin biopsies obtained from patients with SSc compared to healthy tissue samples, which is consistent with our findings. However, the study was limited by its small sample size,

including only eight healthy controls and eight patients with diffuse cutaneous SSc [28].

The aforementioned studies lacked data regarding the potential correlation between HIF-1α levels and specific subtypes of SSc, as well as the evaluation of microcirculation abnormalities observed in capillaroscopy. Thus, our study serves as a significant complement to these prior investigations, enhancing knowledge on associations of HIF-1α with disease activity. We did not find a significant difference in HIF-1α concentration between the limited and diffuse cutaneous SSc groups. However, our findings revealed a significant correlation between elevated HIF-1α levels and microcirculatory dysfunction in patients with SSc, as evidenced by the abnormalities observed in nailfold capillaroscopy. Specifically, patients with the "active" pattern of the disease exhibited significantly higher levels of HIF-1α compared to those with the "early" or "late" pattern.

Vascular damage, which can be observed in capillaroscopy, is an inherent and prominent characteristic in the clinical presentation of SSc [39]. The "early" pattern of SSc is defined by the presence of a limited number of giant capillaries and microhemorrhages, the absence of avascular regions, and a relatively well-preserved capillary distribution. At this stage, loss of capillaries, vascular architectural disorganization, and ramified capillaries are uncommon. The "active" pattern exhibits a significant increase in nailfold capillary aberrations in comparison to the "early" pattern and is characterized by numerous giant capillaries and microhemorrhages, moderate capillary loss (20–30%), and a slightly disorganized capillary architecture with very few branched capillaries. In the "late" pattern a significant absence of giant capillaries and microhemorrhages, extensive avascular regions (with a 50–70% capillary loss), a large number of branched and ramified bushy capillaries (indicative of neoangiogenesis), and complete disarray of the capillary arrangement are seen serving as a hallmark of advanced microcirculation disease [40]. HIF-1α might promote dysregulation of angiogenesis and vasculogenesis, resulting in the abnormalities that can be observed in nailfold capillaries [41].

In contrast to our results, in a study by Ioannou et al. [16], the expression of oxygen-regulated subunit of HIF-1 and VEGF in the skin biopsies in patients with SSc were increased, but there were no statistically significant differences in the expression of HIF-1α and VEGF between patients at different stages of progression (early, active, or late, classified according to capillaroscopic and clinical criteria). In our study, we specifically analyzed blood-circulating HIF-1α levels. The dynamics and interrelation of skin and blood-circulating HIF-1α are still not well defined and require further investigation.

Previous studies examining HIF-1α levels did not analyze the relationship between HIF-1α levels and the existence of ulcers [28, 38]. According to our results, patients with SSc without digital ulcers had significantly higher levels of HIF-1α compared to patients with SSc and either active or healed digital ulcers. This observation, coupled with the finding of elevated HIF-1α levels in patients with SSc and "active" but not "early" or "late" capillaroscopic patterns could indicate the role of this biomarker in identification of patients without the history of ulcers but during acute acceleration in the process of ischemia. We consider this observation to be hypothesis-generating and believe it warrants prospective validation in future studies. The objective would be to identify a subgroup of patients early in the disease stage who have never experienced ulcers but may be at a higher risk of developing them.

Biomarkers related to angiogenesis have been thoroughly investigated in patients with SSc and further explored as potential indicators of organ involvement. Among these biomarkers, VEGF has been the most extensively researched angiogenic mediator. It is a powerful angiogenic factor that encourages the migration, proliferation, and survival of ECs as well as endothelial precursor cells. Distler et al. discovered higher levels of VEGF in patients with SSc who did not have fingertip ulcers, which further suggests a potential protective effect of VEGF [42]. The generation of new blood vessels is crucial in the process of repairing damaged tissues. *VEGF*, a target gene of HIF-α, was considered the most potent endothelial-specific mitogen among those that mediate the process

of vascular remodeling [43]. However, studies have shown that microvascular ECs isolated from patients with SSc have impaired responses to growth factors, including VEGF, which can result in insufficient angiogenesis [44]. VEGF is well known for its role in promoting angiogenesis, and its association with HIF-1α is well established [45]. The hypoxia-inducible factor is a pivotal transcription factor induced under hypoxia which transactivates target genes such as *VEGF* [46]. *VEGF* is a major transcriptional target for HIF-1. Signaling through VEGF receptors has been reviewed [47, 48]. HIF-1 stimulates the production of extracellular matrix fibers, inducing vascular remodeling which leads to abnormal angiogenesis, ultimately resulting in the exacerbation of hypoxia [49].

The serum concentration of HIF-1α seems to be related to the current state of microcirculatory damage. Heger et al. [38] investigated the impact of different treatments on serum HIF-1α levels in patients. The authors conducted a randomized, single-center study to compare the therapeutic outcomes of prostaglandin E_1 (PGE_1) monotherapy versus the co-administration of PGE_1 and an endothelin-1 blocker, bosentan. The authors also found that patients receiving single-drug therapy demonstrated an increase in HIF-1α expression, while patients undergoing combined therapy showed no significant differences in HIF-1α expression with a tendency toward lower expression. An increase in HIF-1α expression in patients treated with only a single drug may be due to disease progression and further deterioration of microcirculation which was prevented in the dual-therapy group [38]. Once again, these findings suggest the potential usefulness of HIF-1α as a biomarker for disease diagnosis and monitoring.

It is important to acknowledge the limitations of our study and interpret the results accordingly. The small number of participants is due to the rarity of the disease and the strict inclusion criteria. However, these criteria were necessary to minimize potential biases by controlling factors that could influence baseline levels of HIF-1α. Replicating these findings in larger patient cohorts is warranted, and further prospective evaluation of HIF-1α in patients at the early stages of the disease would contribute to expanding our knowledge in this field.

CONCLUSION

Vascular abnormalities are important clinical causes of morbidity and mortality in patients with SSc. Our study demonstrates that HIF-1α plasma concentration is significantly elevated in patients with SSc and was able to differentiate between those with "active" from "early" and "late" patterns of vascular damage in capillaroscopy. These findings emphasize the potential clinical utility of HIF-1α in evaluating microcirculatory changes and vascular abnormalities in patients with SSc. This holds promise in aiding the identification of individuals who are still in the early stages of the disease but at risk of disease progression.

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Maciejewska, Mariusz Sikora); writing—review and editing, (Magdalena Maciejewska, Mariusz Sikora, Cezary Maciejewski, Katarzyna Pawlik, Lidia Rudnicka); supervision (Mariusz Sikora and Lidia Rudnicka).

Disclosures. Magdalena Maciejewska, Mariusz Sikora, Albert Stec, Cezary Maciejewski, Katarzyna Pawlik, Michał Zaremba and Lidia Rudnicka have nothing to disclose.

Compliance with Ethics Guidelines. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Bioethical Committee of the Medical University of Warsaw (KB/90/2018 of 21 May 2018). Informed consent was obtained from all subjects involved in the study.

Data Availability. The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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7. OMÓWIENIE PUBLIKACJI STANOWIĄCYCH PRACĘ DOKTORSKĄ

7.1. Chrabaszcz M., Małyszko J., Sikora M., Alda-Malicka R., Stochmal A., Matuszkiewicz-Rowińska J., Rudnicka L. Renal Involvement in Systemic Sclerosis: An Update. *Kidney Blood Press Res.* 2020;45(4):532-548

Celem pracy było zapoznanie się z manifestacjami klinicznymi zaburzonego mikrokrążenia u pacjentów z twardziną układową, do których zaliczamy m.in. zaburzenia czynności nerek. Publikacja powstała we współpracy z jednym z wiodących ośrodków nefrologicznych w Polsce - Kliniką Nefrologii, Dializoterapii i Chorób Wewnętrznych Uniwersyteckiego Centrum Klinicznego Warszawskiego Uniwersytetu Medycznego.

Zaburzenia czynności nerek u pacjentów z twardzina układową mogą mieć różny obraz kliniczny. Jest poważnym czynnikiem pogarszającym rokowanie u chorych na twardzinę układową, a do niedawna było główną przyczyną zgonu w tej chorobie. Wyróżnić możemy m.in.: izolowany zmniejszony wskaźnik filtracji kłębuszkowej (glomerular filtration rate - GFR), kłębuszkowe zapalenie nerek związane z przeciwciałami przeciw cytoplazmie neutrofili, przewlekłą niewydolność nerek, obniżoną funkcję nerek przebiegającą z białkomoczem lub mikroalbuminurią, zwiększoną sztywności tętnic wewnątrznerkowych.

Najbardziej specyficzną i najpoważniejszą chorobą nerek związaną z twardziną układową jest jednak twardzinowy przełom nerkowy *(ang. scleroderma renal crisis – SRC*), który występuje u około 10% pacjentów. Charakteryzuje się szybko postępującą niewydolnością nerek, podwyższonym stężeniem reniny i nagłym wystąpieniem nadciśnienia tętniczego. Leczenie twardzinowego przełomu nerkowego zostało zrewolucjonizowane przez wprowadzenie inhibitorów konwertazy angiotensyny. Jednak nadal około połowa chorych z twardzinowym przełomem nerkowym wymaga dializoterapii, a śmiertelność osiąga 10%. Dlatego trwają badania nad ustaleniem markerów chorobowych klinicznych, ultrasonograficznych oraz serologicznych.

Wczesne rozpoznanie i leczenie nieprawidłowości naczyniowych w twardzinie układowej ma zasadnicze znaczenie dla poprawy wyników leczenia pacjentów i zapobiegania długotrwałym powikłaniom.

7.2. Maciejewska M., Sikora M., Maciejewski C., Alda-Malicka R., Czuwara, J., Rudnicka L., Raynaud's phenomenon with focus on systemic sclerosis. *J. Clin. Med.* 2022, *11*, 2490

W przebiegu twardziny układowej dochodzi do zaburzenia struktury i funkcji naczyń krwionośnych. Uszkodzeniu ulegają małe tętnice, tętniczki i naczynia włosowate, co prowadzi do występowania objawu Raynauda. Objaw ten jest jednym z pierwszych objawów choroby i występuje u większości pacjentów z twardziną układową. Manifestuje się jako bladość, zsinienie lub zaczerwienienie skóry, a także jako uczucie drętwienia lub bólu w obrębie kończyn, zwłaszcza palców. Może objawiać się również na wargach, czubku nosa i małżowinach usznych.

Objaw Raynauda może wystąpić w dwóch różnych formach: pierwotnej i wtórnej. Objaw Raynauda pierwotny (*ang. primary Raynaud's phenomenon*) występuje samoistnie i nie jest związane z innymi chorobami. Objaw Raynauda wtórny (*ang. secondary Raynaud's phenomenon*) występuje u pacjentów z innymi chorobami, takimi jak twardzina układowa. Ponadto, objaw Raynauda wtórny może prowadzić do poważniejszych powikłań i wymaga bardziej intensywnego leczenia niż objaw Raynauda pierwotny.

Nieprawidłowości naczyniowe obserwowane w twardzinie układowej można uwidocznić poprzez przeprowadzenie kapilaroskopii. Kapilary wałów paznokciowych łatwo uwidocznić podczas badania, ponieważ ich oś długa przebiega równolegle. Mikroangiopatia typowa dla twardziny układowej to strukturalne zmiany naczyń włosowatych, które występują u większości pacjentów już we wczesnym stadium choroby. Standardem jest klasyfikowanie zmian kapilaroskopowych wg wzoru Cutolo dzieląc je na zmiany o typie wczesnym, aktywnym oraz późnym. W fazie wczesnej zmiany kapilaroskopowe charakteryzują się nielicznymi poszerzonymi pętlami naczyniowymi. Faza aktywna to liczne megakapilary, wybroczyny oraz redukcja pętli naczyniowych. Natomiast dla fazy późnej typowe są rozgałęzione kapilary i obszary pozbawione naczyń. Zmiany naczyniowe odzwierciedlają stopień zaawansowania twardziny układowej, pozwalają potwierdzić rozpoznanie, a następnie monitorować postęp choroby.

Badanie kapilaroskopowe wykonywałam u pacjentów z twardziną układową, podczas oceny zaburzonego mikrokrążenia obwodowego w badaniu stanowiącym rozprawę doktorską.

7.3. Maciejewska M., Stec A., Zaremba M., Maciejewski C., Rudnicka L., Sikora M Copeptin as a biomarker of microcirculation alterations in systemic sclerosis *Clin Cosmet Investig Dermatol.* 2023;16:1351-1361

Wciąż trwają poszukiwania biomarkerów szybkiej progresji zmian naczyniowych, które odpowiednio wcześnie pozwoliłyby na intensyfikację leczenia reologicznego.

Celem badania było oznaczenie stężenia kopeptyny (peptyd określający aktywność układu wazopresynergicznego, który ma silne właściwości naczynioskurczowe) u pacjentów z twardziną układową. Uszkodzenie naczyń jest nieodłączną i często dominującą cechą obrazu klinicznego twardziny układowej. Krytyczne niedokrwienie wraz z owrzodzeniami dystalnych części palców stanowi najcięższą postać zaburzeń mikrokrążenia, prowadząc do wystąpienia bólu, niepełnosprawności oraz zmniejszenia jakości życia. Kopeptyna jest C-końcową częścią pro-wazopresyny. Jej cechy, takie jak stabilność w osoczu, łatwość zmierzenia oraz dłuższy okres półtrwania niż wazopresyny sprawiają, że jest ona coraz szerzej badaną cząsteczką dla określenia aktywności układu wazopresynergicznego w organizmie. Wazopresyna jest ważnym peptydem o właściwościach wazokonstrykcyjnych, który może podobnie do angiotensyny II i endoteliny, być zaangażowanym w skurcz naczyń podczas objawu Raynauda oraz uszkodzenie komórek śródbłonka

Do badania zakwalifikowano 34 pacjentów z twardziną układową oraz 30 ochotników stanowiących dobraną pod względem wieku i płci grupę kontrolną. Stężenie kopeptyny w surowicy krwi oznaczono metodą immunoenzymatyczną (ELISA).

Pacjenci z twardziną układową mieli znacznie wyższe stężenie kopeptyny w osoczu (4,21 pmol/L [3,04-5,42]) w porównaniu do grupy kontrolnej (3,40 pmol/L [2,38-3,76], $p<0,01$). Stwierdzono dodatnią korelację pomiędzy nasileniem objawu Raynauda a stężeniem kopeptyny ($r=0,801$, $p<0,05$). Pacjenci „późnym" wzorcem zmian naczyniowych w kapilaroskopii (5,37 pmol/l [4,29-8,06]) charakteryzowali się wyższymi wartościami kopeptyny niż pacjenci o „wczesnym" (2,43 pmol/l [2,25-3,20], $p<0,05$) i aktywnym wzorcu (3,93 pmol/l [2,92-5,16], $p<0,05$). Pacjenci z twardzina układową, u których występowały owrzodzenia paliczków palców mieli istotnie statystycznie wyższe stężenie kopeptyny (5,71 pmol/l [IQR 4,85-8,06]) niż pacjenci bez owrzodzeń na paliczkach palców (3,31 pmol/l, [2,28-4,30], $p<0,05$). Ponadto stężenie kopeptyny charakteryzowało się dobrą dokładnością diagnostyczną w rozróżnianiu pacjentów z owrzodzeniem palców i bez (AUC=0,863). Dożylne leczenie analogami prostaglandyn (alprostadylem)

powodowało zmniejszenie stężenia kopeptyny z 4,96 [4,02-6,01] do 3,86 pmol/l [3,17-4,63] ($p<0,01$) po 4-6 cyklach podawania.

Kopeptyna wydaje się obiecującym biomarkerem uszkodzenia naczyniowego w twardzinie układowej i odpowiedzi na leczenie poprawiające właściwości reologiczne krwi. Dalsze badania nad kopeptyną mogą przyczynić się do identyfikacji pacjentów o szybko postępujących zmianach naczyniowych i wysokim ryzyku powstania owrzodzeń palców. Wyniki badań mogą przyczynić się do znalezienia biomarkerów pozwalających monitorować aktywność twardziny układowej, ocenić rokowanie pacjenta i przewidzieć odpowiedź na leczenie.

7.4. Maciejewska M., Sikora M., Stec A., Pawlik K., Zaremba M., Rudnicka L. Hypoxia-inducible factor-1α (HIF-1α) as a biomarker for changes in microcirculation in individuals with systemic sclerosis. ***Dermatol Ther (Heidelb) 2023*** **https://doi.org/10.1007/s13555-023-00952-w**

W przebiegu twardziny układowej na wczesnym etapie dochodzi do uszkodzenia śródbłonka, co sprzyja przewlekłemu niedokrwieniu skóry oraz innych narządów. Rosnąca liczba badań wskazuje, że czynnik indukowany hipoksją-1 α (Hypoxia-inducible factor-1α (HIF-1α), kluczowy czynnik transkrypcyjny w odpowiedzi na przewlekłą hipoksję, może mieć istotne znaczenie w patogenezie chorób przebiegających z nadmiernym włóknieniem Biorąc pod uwagę plejotropowe działania HIF-1α w twardzinie układowej, m.in. wpływ na angiogenezę, proliferację i akumulację włókien macierzy zewnątrzkomórkowej, cząsteczka może okazać się dobrym biomarkerem i punktem uchwytu nowych opcji terapeutycznych.

Celem badania było określenie związku pomiędzy stężeniem czynnika indukowanego hipoksją-1 α u pacjentów z twardziną układową a aktywnością choroby i nasileniem zaburzeń mikrokrążenia obwodowego.

Do badania zakwalifikowano 50 pacjentów z twardziną układową oraz 30 zdrowych ochotników stanowiących dobraną pod względem wieku i płci grupę kontrolną. Stężenie stężenie czynnika indukowanego hipoksją-1α w surowicy krwi oznaczono metodą immunoenzymatyczną (ELISA).

Oceny klinicznej zajęcia naczyń krwionośnych dokonano poprzez ocenę nasilenia objawu Raynauda ocenione w skali Raynaud's Condition Score (RCS), ocenę występowania czynnych i zagojonych owrzodzeń paliczków, teleangiektazji. U każdego pacjenta wykonano również badanie kapilaroskopowe wału paznokciowego ocenione

w skali Cutolo (wzór wczesny, aktywny lub późny). Aktywność choroby oceniono za pomocą wskaźnika EUSTAR Revised Activity Index.

Pacjenci z twardziną układową mieli istotnie statystycznie wyższe stężenie czynnika indukowanego hipoksją-1α (3,042 ng/ml [2,295-7,749]) w porównaniu z grupą kontrolną (1,969 ng/ml [1,531-2,903] p<0,01). Pacjenci z uogólnioną postacią twardziny układowej (2,803 ng/ml, IQR 2,221-8,799) i formą ograniczoną (3,231 ng/ml, IQR 2,566-5,502) wykazywali zwiększone stężenie czynnika indukowanego hipoksją-1α w porównaniu z grupą kontrolną (p<0,01). Stwierdzono statystycznie wyższe stężenie czynnika indukowanego hipoksją-1α u pacjentów z "aktywnym" kapilaroskopowym obrazem twardzinowym wg klasyfikacji Cutolo (6,625 ng/ml, IQR 2,488-11,480) w porównaniu z pacjentami z obrazem „wczesnym" (2,739, IQR 2,165-3,282, p<0,05) i „późnym" (2,983 ng/ml, IQR 2,229-3,386, p<0,05). Jednocześnie stwierdzono, że pacjenci, u których nie występowały owrzodzenia paliczków palców mieli znacząco wyższe stężenie czynnika indukowanego hipoksją-1α (4,367 ng/ml, IQR 2,488-9,462) niż pacjenci z aktywnymi owrzodzeniami paliczków palców (2,832 ng/ml, IQR 2,630-3,094, p< 0,05) oraz owrzodzeniami paliczków palców w przeszłości (2,668 ng/ml, IQR - 2,074-2,983, p<0,05).

8. WNIOSKI

1. Kopeptyna i czynnik indukowany hipoksją-1α (HIF-1α) mogą posłużyć jako potencjalne biomarkery zaburzeń mikrokrążenia obwodowego u pacjentów z twardziną układową
2. Stężenie kopeptyny w surowicy wykazuje pozytywną korelację z nasileniem objawu Raynauda i występowaniem owrzodzeń obwodowych w przebiegu twardziny układowej oraz jest czułym parametrem odpowiedzi na leczenie reologiczne
3. Stwierdzono statystycznie wyższe stężenie czynnika indukowanego hipoksją-1α w surowicy u pacjentów z "aktywnym" kapilaroskopowym obrazem twardzinowym wg klasyfikacji Cutolo w porównaniu z pacjentami z obrazem „wczesnym" i „późnym"
4. Monitorowanie stężenia kopeptyny i czynnika indukowanego hipoksją-1α u pacjentów z twardziną układową może pozwolić na identyfikację pacjentów o wysokim ryzyku nasilonych zaburzeń mikrokrążenia i tym samym wczesnego rozpoczęcia intensywnego leczenia reologicznego

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dotyczącym: wyrażenia opinii w sprawie badania pt :„ Rola czynnika indukowanego hipoksją 1 (HIF-1) w patogenezie zaburzeń naczyniowych w przebiegu twardziny układoweji."

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- ~~stwierdza, że jest ono niedopuszczalne i niezgodne z zasadami naukowo-etycznymi.*~~

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dotyczącym: wyrażenia opinii w sprawie badania pt. „Nowe biomarkery nasilenia zaburzeń mikrokrążenia obwodowego w twardzinie układowej"

- Badanie może być prowadzone wyłącznie w okresie obowiązywania polisy ubezpieczeniowej.

wyraża następującą
opinię

- stwierdza, że jest ono dopuszczalne i zgodne z zasadami naukowo-etycznymi*.
- ~~stwierdza, że jest ono niedopuszczalne i niezgodne z zasadami naukowo-etycznymi.*~~

Uwagi Komisji – *verte*

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Komisja działa zgodnie z zasadami GCP .

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Publikacja 1

Raynaud's phenomenon with focus on systemic sclerosis. Maciejewska, M.; Sikora, M.; Maciejewski, C.; Alda-Malicka, R.; Czuwara, J.; Rudnicka, L. *J. Clin. Med.* 2022, *11*, 2490.

Lp.	Autor	Rodzaj wkładu merytorycznego	Procentowy wkład w procesie publikacji	Data, podpis
1.	lek. Magdalena Maciejewska	• stworzenie projektu badania • gromadzenie danych • opracowanie rycin i tabeli • opracowanie artykułu	65%	15/06/2023
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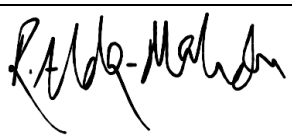
Publikacja nr 2

Renal Involvement in Systemic Sclerosis: An Update. Kidney Blood Press Res. Chrabaszcz M, Małyszko J, Sikora M, Alda-Malicka R, Stochmal A, Matuszkiewicz-Rowinska J, Rudnicka L. 2020;45(4):532-548. doi: 10.1159/000507886. Epub 2020 Jun 10. PMID: 32521536.

Lp.	Autor	Rodzaj wkładu merytorycznego	Procentowy wkład w procesie publikacji	Data, podpis
1.	lek. Magdalena Maciejewska	• stworzenie projektu badania • gromadzenie danych • opracowanie rycin i tabeli • opracowanie artykułu	~~50%~~ 55 %	15/06/2023 Magdalena Maciejewska
2.	prof. dr hab. n. med. Jolanta Małyszko	• rewizja artykułu • stworzenie projektu badania	10%	J. Małyszko
3.	dr n. med. Mariusz Sikora	• rewizja artykułu • stworzenie projektu badania	10%	12/06/2023
4.	Rosanna Alda-Malicka	• rewizja artykułu	5%	
5.	dr n. med. Anna Stochmal	• rewizja artykułu	5%	Anna Niemczyk (Stochmal) 12.06.2023
6.	prof. dr hab. n. med. Joanna Matuszkiewicz-Rowinska	• rewizja artykułu • stworzenie projektu badania	~~10%~~ 5%	
7.	prof. dr hab. n. med. Lidia Rudnicka	• stworzenie projektu badania • rewizja artykułu	~~10%~~ 5 %	

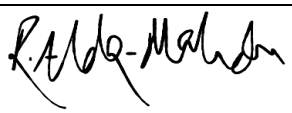
Publikacja 1

Raynaud's phenomenon with focus on systemic sclerosis. Maciejewska, M.; Sikora, M.; Maciejewski, C.; Alda-Malicka, R.; Czuwara, J.; Rudnicka, L. *J. Clin. Med.* 2022, *11*, 2490.

Lp.	Autor	Rodzaj wkładu merytorycznego	Procentowy wkład w procesie publikacji	Data, podpis
1.	lek. Magdalena Maciejewska	• stworzenie projektu badania • gromadzenie danych • opracowanie rycin i tabeli • opracowanie artykułu	65%	
2.	dr n. med. Mariusz Sikora	• rewizja artykułu • stworzenie projektu badania	10%	
3.	lek. Cezary Maciejewski	• rewizja artykułu	5%	
4.	lek. Rosanna Alda-Malicka	• rewizja artykułu	5%	 12/01/2023
5.	dr hab. n. med. Joanna Czuwara	• rewizja artykułu	5%	
6.	prof. dr hab. n. med. Lidia Rudnicka	• stworzenie projektu badania • rewizja artykułu	10%	

Publikacja nr 2

Renal Involvement in Systemic Sclerosis: An Update. Kidney Blood Press Res. Chrabaszcz M, Małyszko J, Sikora M, Alda-Malicka R, Stochmal A, Matuszkiewicz-Rowinska J, Rudnicka L. 2020;45(4):532-548. doi: 10.1159/000507886. Epub 2020 Jun 10. PMID: 32521536.

Lp.	Autor	Rodzaj wkładu merytorycznego	Procentowy wkład w procesie publikacji	Data, podpis
1.	lek. Magdalena Maciejewska	• stworzenie projektu badania • gromadzenie danych • opracowanie rycin i tabeli • opracowanie artykułu	55%	
2.	prof. dr hab. n. med. Jolanta Małyszko	• stworzenie projektu badania • rewizja artykułu	10%	
3.	dr n. med. Mariusz Sikora	• stworzenie projektu badania • rewizja artykułu	10%	
4.	Rosanna Alda-Malicka	• rewizja artykułu	5%	 12/01/2023
5.	dr n. med. Anna Stochmal	• rewizja artykułu	5%	
6.	prof. dr hab. n. med. Joanna Matuszkiewicz-Rowinska	• rewizja artykułu • stworzenie projektu badania	10%	
7.	prof. dr hab. n. med. Lidia Rudnicka	• stworzenie projektu badania • rewizja artykułu	5%	

Publikacja nr 3

Maciejewska M., Stec A., Zaremba M., Maciejewski C., Rudnicka L., Sikora M Copeptin as a biomarker of microcirculation alterations in systemic sclerosis *Clin Cosmet Investig Dermatol*. 2023;16:1351-1361

Lp.	Autor	Rodzaj wkładu merytorycznego	Procentowy wkład w procesie publikacji	Data, podpis
1.	lek. Magdalena Maciejewska	• stworzenie projektu badania • gromadzenie danych • opracowanie artykułu	65%	15/06/2023
2.	lek. Albert Stec	• rewizja artykułu • gromadzenie danych	5%	14.06.2023
3.	mrg Michał Zaremba	• gromadzenie danych	5%	14.06.2023
4.	lek. Cezary Maciejewski	• rewizja artykułu	5%	12/06/2023
5.	prof. dr hab. n. med. Lidia Rudnicka	• stworzenie projektu badania • rewizja artykułu	10%	
6.	dr n. med. Mariusz Sikora	• stworzenie projektu badania • gromadzenie danych • analiza statystyczna	10%	12/06/2023

Publikacja nr 4

Maciejewska M., Sikora M., Stec A., Pawlik K., Zaremba M., Rudnicka L. Hypoxia-inducible factor-1α (HIF-1α) as a biomarker for changes in microcirculation in individuals with systemic sclerosis. *Dermatology and Therapy 2023 (przyjęta do druku)*

Lp.	Autor	Rodzaj wkładu merytorycznego	Procentowy wkład w procesie publikacji	Data, podpis
1.	lek. Magdalena Maciejewska	• stworzenie projektu badania • gromadzenie danych • opracowanie rycin i tabeli • opracowanie artykułu	60%	15/01/2023
2.	dr n. med. Mariusz Sikora	• stworzenie projektu badania • rewizja artykułu • analiza statystyczna	10%	12/06/2023
3.	lek. Albert Stec	• gromadzenie danych • rewizja artykułu	5%	14.06.2023
4.	mgr Michał Zaremba	• gromadzenie danych	5%	14.06.2023
5.	lek. Cezary Maciejewski	• rewizja artykułu	5%	12/06/2023
6.	lek. Katarzyna Pawlik	• rewizja artykułu	5%	9.06.2023
7.	prof. dr hab. n. med. Lidia Rudnicka	• stworzenie projektu badania • rewizja artykułu	10%	