

lek. Damian Sieńko

Wpływ wybranych parametrów na metabolizm ludzkich  
mezenchymalnych komórek macierzystych oraz ich potencjał  
regeneracyjny w terapii leczenia ran.

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

Promotor: dr hab. n. med. Beata Mrozikiewicz-Rakowska, prof. CMKP

Klinika Diabetologii i Chorób Wewnętrznych  
Warszawski Uniwersytet Medyczny



Obrona rozprawy doktorskiej przed Radą Dyscypliny Nauk Medycznych  
Warszawskiego Uniwersytetu Medycznego

Warszawa, 2025 r.



**Słowa kluczowe:** ludzkie mezenchymalne komórki macierzyste; zespół stopy cukrzycowej; statyny; hipoksja; terapia komórkowa

**Key words:** human mesenchymal stromal cells; diabetic foot ulcer; statins; hypoxia; cell therapy

Badania wchodzące w skład niniejszej pracy doktorskiej zostały przeprowadzone w ramach:

Praca nr 1 została zrealizowana w ramach projektu badawczego pt. *Ocena wpływu atorwastatyny i rosuwastatyny na wybrane parametry ludzkich mezenchymalnych komórek macierzystych* sfinansowanego z mini-grantu studenckiego Warszawskiego Uniwersytetu Medycznego o numerze 1W21/NM1/17.

Prace nr 2 oraz 3 zostały zrealizowane w ramach grantu o numerze:

STRATEGMED2/267976/13/NCBR/2015 sfinansowanego przez Narodowe Centrum Badań i Rozwoju.



*Najserdeczniejsze podziękowania składam:*

*Mojej Promotor, Pani Profesor Beacie Mrozikiewicz-Rakowskiej za nieocenione wsparcie, cenne wskazówki i życzliwość okazywaną na każdym etapie powstawania niniejszej rozprawy.*

*Panu Profesorowi Leszkowi Czupryniakowi, którego wiedza i zaangażowanie były dla mnie nieocenioną pomocą.*

*Współautorom artykułów składających się na niniejszą rozprawę doktorską za wkład merytoryczny i naukowy, bez którego realizacja pracy nie byłaby możliwa.*

*Żonie za cierpliwość, wyrozumiałość i wsparcie w chwilach zwątpienia.*

*Rodzicom, którzy swoim zaangażowaniem, wiarą i nieustanną motywacją przyczynili się do pomyślnego ukończenia tej rozprawy.*



### Wykaz wszystkich publikacji

| Rodzaj publikacji                                                  | Liczba | Impact Factor | Punkty MNiSW |
|--------------------------------------------------------------------|--------|---------------|--------------|
| Prace włączone do<br>rozprawy<br>doktorskiej                       | 3      | 12,266        | 320          |
| Prace, które nie<br>zostały włączone<br>do rozprawy<br>doktorskiej | 7      | 11,312        | 480          |
| Razem                                                              | 10     | 23,578        | 800          |

## Wykaz publikacji stanowiących rozprawę doktorską:

- 1) **The influence of oxygen deprivation and donor age on the effect of statins on human mesenchymal stromal cells.**

*Sieńko Damian, Klimczak-Tomaniak Dominika, Kulesza Agnieszka, Symonides Helena, Kuch Marek, Pączek Leszek, Burdzińska Anna*; Tissue and Cell; 2020; Praca oryginalna (IF= 2,466 MNiSW: 40)

- 2) **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact.**

*Mrozikiewicz-Rakowska Beata, Szablowska-Gadomska Iлона, Cysewski Dominik, Rudziński Stefan, Płoski Rafał, Gasperowicz Piotr, Konarzewska Magdalena, Zieliński Jakub, Mieczkowski Mateusz, Sieńko Damian, Grzela Tomasz, Noszczyk Maria, Paleska Barbara, Czupryniak Leszek, Lewandowska-Szumieł Małgorzata*; International Journal of Molecular Sciences; 2023; Praca oryginalna (IF= 4,9 MNiSW: 140)

- 3) **The Potential of Mesenchymal Stem/Stromal Cells in Diabetic Wounds and Future Directions for Research and Therapy-Is It Time for Use in Everyday Practice?**

*Sieńko Damian, Szablowska-Gadomska Iлона, Nowak-Szwed Anna, Rudziński Stefan, Gofron Maksymilian, Zygmunciak Przemysław, Lewandowska-Szumieł Małgorzata, Zgliczyński Wojciech Stanisław, Czupryniak Leszek, Mrozikiewicz-Rakowska Beata*; International Journal of Molecular Sciences; 2024; Praca pogładowa (IF=4,9 MNiSW: 140)

Łącznie:

IF: 12,266

MNiSW: 320

## Spis treści

|                                                                                                                                                                          |    |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 1. Wykaz zastosowanych skrótów .....                                                                                                                                     | 11 |
| 2. Streszczenie w języku polskim .....                                                                                                                                   | 13 |
| 3. Streszczenie w języku angielskim .....                                                                                                                                | 18 |
| 4. Wstęp uzasadniający połączenie wskazanych publikacji w jeden cykl oraz<br>komentujący osiągnięcia naukowe na tle dotychczasowego stanu wiedzy .....                   | 23 |
| 5. Założenia i cel pracy .....                                                                                                                                           | 28 |
| 6. Kopie opublikowanych prac .....                                                                                                                                       | 29 |
| 6.1. The influence of oxygen deprivation and donor age on the effect of statins....                                                                                      | 29 |
| on human mesenchymal stromal cells .....                                                                                                                                 | 29 |
| 6.2. Allogenic Adipose-Derived stem cells in diabetic foot ulcer treatment: Clinical<br>effectiveness, safety, survival in the Wound site, and proteomic impact.....     | 36 |
| 6.3. The potential of mesenchymal stem.stromal cells in diabetic wounds and future<br>directions for reasearch and therapy-is it time for use in everyday practice?..... | 52 |
| 7. Podsumowanie .....                                                                                                                                                    | 71 |
| 8. Wnioski .....                                                                                                                                                         | 73 |
| 9. Bibliografia .....                                                                                                                                                    | 74 |
| 10. Oświadczenia współautorów .....                                                                                                                                      | 76 |



## 1. Wykaz zastosowanych skrótów

|                |                                                                                                               |
|----------------|---------------------------------------------------------------------------------------------------------------|
| <b>ADSC</b>    | mezenchymalne komórki macierzyste izolowane z tkanki tłuszczowej<br>(ang. adipose-derived stem cells)         |
| <b>AGE</b>     | późne produkty glikacji<br>(ang. advanced glycation endproducts)                                              |
| <b>BM-MSC</b>  | mezenchymalne komórki macierzyste pozyskane ze szpiku kostnego<br>(ang. bone marrow mesenchymal stem cells)   |
| <b>BrdU</b>    | 5-bromo-2'deoksyurydyna<br>(ang. 5-bromo-2'-deoxyuridine)                                                     |
| <b>CAT</b>     | ang. human catalase enzyme                                                                                    |
| <b>DFU</b>     | zespół stopy cukrzycowej (ang. diabetic foot ulcer)                                                           |
| <b>FGF</b>     | czynnik wzrostu fibroblastów<br>(ang. fibroblast growth factor)                                               |
| <b>GAPDH</b>   | dehydrogenaza aldehydu 3-fosfoglicerynowego<br>(ang. glyceraldehyde 3-phosphate dehydrogenase)                |
| <b>HGF</b>     | czynnik wzrostu hepatocytów<br>(ang. hepatocyte growth factor)                                                |
| <b>HMG-CoA</b> | 3-hydroksy-3-metyloglutarylokoenzym A<br>(ang. 3-hydroxy-3methylglutaryl coenzyme A)                          |
| <b>IDF</b>     | Międzynarodowa Federacja Diabetologiczna<br>(ang. International Diabetes Federation)                          |
| <b>IWGDF</b>   | Międzynarodowa Grupa Robocza ds. Stopy Cukrzycowej<br>(ang. International Working Group on the Diabetic Foot) |
| <b>LDH</b>     | dehydrogenaza mleczanowa<br>(ang. lactate dehydrogenase)                                                      |
| <b>MSC</b>     | mezenchymalne komórki macierzyste<br>(ang. mesenchymal stem cells)                                            |
| <b>VEGF</b>    | czynnik wzrostu śródbłónka naczyniowego<br>(ang. vascular endothelial growth factor)                          |
| <b>ZSC</b>     | zespół stopy cukrzycowej                                                                                      |





## 2. Streszczenie w języku polskim

Tytuł: *Wpływ wybranych parametrów na metabolizm ludzkich mezenchymalnych komórek macierzystych oraz ich potencjał regeneracyjny w terapii leczenia ran*

### Wstęp

Celem niniejszej rozprawy doktorskiej jest kompleksowa analiza wpływu wybranych warunków hodowli in vitro na metabolizm ludzkich mezenchymalnych komórek macierzystych (MSC) oraz ocena ich skuteczności terapeutycznej w klinicznym modelu trudno gojących się ran na przykładzie zespołu stopy cukrzycowej (ZSC). W obliczu dynamicznego rozwoju medycyny regeneracyjnej oraz potrzeby opracowania skutecznych metod wspomagających procesy naprawcze tkanek, kluczowe staje się poznanie mechanizmów warunkujących korzystne działanie MSC na regenerację uszkodzeń tkankowych.

MSC charakteryzuje zdolność samoodnowy, wielokierunkowego różnicowania (w kierunku osteoblastów, chondrocytów czy adipocytów) oraz efektywne działanie parakryne, przejawiające się modulacją odpowiedzi zapalnej oraz pobudzaniem angiogenezy. Dotychczasowe wyniki badań wskazują na potencjalne zastosowanie statyn, leków o właściwościach plejotropowych, mogących wzmacniać funkcje MSC w określonych warunkach środowiskowych. Dodatkowo istotnym czynnikiem modulującym metabolizm, proliferację i różnicowanie MSC jest stężenie tlenu w środowisku hodowli (normoksja versus hipoksja).

Zrozumienie mechanizmów prowadzących do poprawy gojenia ran w warunkach zwiększonych wymagań metabolicznych oraz w środowisku zaburzonej perfuzji (jak w cukrzycy) jest nieodzowne do wdrożenia innowacyjnych terapii w codziennej praktyce klinicznej. Niniejszy cykl publikacji przyczynia się do poszerzenia wiedzy o działaniu statyn oraz hipoksji na MSC, a także o protokoły kliniczne stosowania MSC w trudno gojących się ranach.

Dotychczas żadna praca naukowa nie oceniała łącznego wpływu statyn oraz ograniczenia stężenia tlenu w ramach hodowli MSC. Przedmiotem pracy nr 1 był wpływ atorwastatyny oraz rosuwastatyny na MSC w zróżnicowanych warunkach tlenowych, również z uwzględnieniem wieku dawców. W pracy nr 2 przeprowadzono badania in vivo:

mezenchymalne komórki macierzyste, izolowane z tkanki tłuszczowej (ADSC), aplikowano do ran w obrębie stopy cukrzycowej, w celu weryfikacji skuteczności terapii komórkowej w leczeniu trudno gojących się ran. W pracy nr 3 dokonano przeglądu literatury i wyników dotychczasowych badań, aby ułatwić przyszłym badaczom optymalne projektowanie terapii komórkowych przy leczeniu ran w stanach takich jak zespół stopy cukrzycowej czy inne przewlekłe owrzodzenia.

### **Materialy i metody**

W pierwszym etapie badań wykorzystano ludzkie mezenchymalne komórki macierzyste pozyskane ze szpiku kostnego (BM-MSC), które pobrano od 12 pacjentów po uzyskaniu ich pisemnej zgody. Badanie rozpoczęto po uzyskaniu zgody Lokalnej Komisji Bioetycznej. Zostało ono przeprowadzone w Klinice Immunologii, Transplantologii i Chorób Wewnętrznych Warszawskiego Uniwersytetu Medycznego. Przeprowadzono standardową charakterystykę fenotypową komórek (ekspresja antygenów CD73, CD90, CD105 oraz brak ekspresji m.in. CD45, CD34) oraz poddano je testom różnicowania do linii osteogenicznej, chondrogenicznej i adipogenicznej.

Dla oceny wpływu warunków tlenowych zastosowano dwa środowiska hodowli. W pierwszej grupie hodowli zastosowano środowisko normoksyjne (21% O<sub>2</sub>, standardowe warunki laboratoryjne), natomiast w drugiej środowisko hipoksyjne (2% O<sub>2</sub>, odzwierciedlające środowisko niedotlenienia komórek). Komórki inkubowano z atorwastatyną w stężeniu 0,05 µM (zbliżonym do fizjologicznego stężenia terapeutycznego u pacjentów) oraz z rosuwastatyną w stężeniu 0,04 µM. Dodatkowo przeprowadzono testy cytotoksyczności z użyciem niższych stężeń oraz stężenia 1 µM. Badano proliferację metodą BrdU (5-bromo-2'-deoksyurydyna) oraz cytotoksyczność za pomocą aktywności dehydrogenazy mleczanowej (LDH) w podłożu.

Praca nr 2 podsumowuje badanie kliniczne z udziałem pacjentów z trudno gojącymi się ranami zespołu stopy cukrzycowej. Badanie rozpoczęto po uzyskaniu zgody Komisji Bioetycznej (KB/128/2019 24.06.2019 i KB/3/A2021 18.01.2021). Zostało ono zarejestrowane w zbiorze ClinicalTrials.gov pod numerem NCT03865394. Grupa badana obejmowała 47 pacjentów podzielonych na dwie podgrupy: pacjentów otrzymujących ADSC w fibrynowym nośniku lub otrzymujących sam fibrynowy klej tkankowy (grupa kontrolna). ADSC izolowane z tkanki tłuszczowej zdrowych dawców aplikowano

na powierzchnię ran, monitorując następnie przebieg gojenia oraz czas potrzebny do uzyskania 50-procentowej redukcji powierzchni owrzodzenia. Ponadto w materiale pobieranym z rany przeprowadzono analizy proteomiczne, a także ocenę obecności DNA pochodzącego od dawców komórek, w celu zweryfikowania ich czasu przeżycia w miejscu podania.

Praca nr 3 stanowi przegląd literatury naukowej, w którym autorzy podsumowują:

- mechanizmy działania MSC (m.in. efekty parakryne, immunomodulacja),
- wyniki najważniejszych badań przedklinicznych i klinicznych z zastosowaniem MSC,
- potencjalne modyfikacje protokołów terapeutycznych, takie jak zastosowanie prekondycjonowania komórek macierzystych, modyfikacje genetyczne czy wykorzystanie rusztowań wspomagających przeżycie i integrację komórek w ranie,
- ograniczenia i perspektywy rozwoju terapii MSC w leczeniu ran przewlekłych, zwłaszcza w cukrzycy.

W przeglądzie zwrócono również uwagę na kwestie bezpieczeństwa stosowania MSC, ze szczególnym uwzględnieniem ryzyka immunogenności w warunkach allogenicznych oraz możliwych reakcji zapalnych.

## **Wyniki**

Obserwacje poczynione w pracy nr 1 określają optymalne warunki prekondycjonowania przy użyciu statyn oraz zmiennego stężenia tlenu dla najbardziej efektywnego działania MSC.

Wykazano, że:

- atorwastatyna w stężeniach zbliżonych do fizjologicznych istotnie zwiększała proliferację MSC, zarówno w warunkach normoksji, jak i hipoksji, nie wywołując przy tym znaczącej cytotoksyczności;
- rosuwastatyna w warunkach hipoksji również stymulowała proliferację bez istotnych efektów toksycznych, natomiast w normoksji nieco częściej powodowała niewielkie wzrosty cytotoksyczności (zależne od stężenia leku);
- hipoksja *per se* zwiększała zdolności proliferacyjne MSC, co może oznaczać, że komórki w warunkach niskiego stężenia tlenu lepiej adaptują się do środowiska z jego ograniczonym dostępem (np. w tkankach niedokrwionych);

- wiek dawców nie wykazał w analizach istotnego różnicującego wpływu na odpowiedź komórkową w kontekście statyn w testowanych populacjach (choć ogólnie starszy wiek dawców może korelować ze zmniejszoną aktywnością proliferacyjną MSC).

W pracy nr 2 zaobserwowano następujące wyniki wielotygodniowej obserwacji pacjentów z ZSC:

- zmniejszenie powierzchni rany następowało szybciej w grupie otrzymującej ADSC niż w grupie kontrolnej, co potwierdzono zarówno krótszym czasem do redukcji powierzchni owrzodzenia o 50%, jak i większym odsetkiem pełnego wygojenia do końca obserwacji;
- analiza proteomiczna wskazała na wzmożoną ekspresję białek odpowiadających za procesy regeneracyjne i angiogenezę (GAPDH, CAT i inne)
- wykrywalność DNA dawców w miejscu rany utrzymywała się do około 2-3 tygodni od aplikacji MSC; co sugeruje, że allogeniczne MSC mogą przetrwać w środowisku rany wystarczająco długo, by zainicjować korzystne zmiany w przebiegu gojenia;
- nie zanotowano poważnych zdarzeń niepożądanych bezpośrednio związanych z podaniem MSC, co przemawia za bezpieczeństwem stosowania takiej terapii w warunkach klinicznych.

Wyniki badań 1 i 2 miały znaczący wpływ na powstanie pracy poglądowej (praca nr 3), która podsumowuje dotychczasowe obserwacje i nakreśla kierunki dalszych badań nad wykorzystaniem komórek macierzystych w terapii leczenia ran:

- mechanizm działania MSC w ranach obejmuje immunomodulację (m.in. zmiana profilu cytokin pro- i przeciwzapalnych), stymulację angiogenezy (poprzez sekrecję czynników wzrostu, np. VEGF) oraz bezpośrednie wspomaganie procesu tworzenia nowej tkanki;
- wpływ parametrów hodowli (stężenie tlenu, obecność leków plejotropowych, wiek dawcy itp.) może optymalizować terapeutyczny potencjał MSC;
- strategie usprawniania terapii: modyfikacje genetyczne, aktywacja MSC bodźcami (np. hipoksja, czynniki wzrostu), łączenie komórek z rusztowaniami (np. hydrożele czy konstrukty tkankowe);
- zagadnienia bezpieczeństwa i standaryzacja: szczególną uwagę należy zwrócić na czystość mikrobiologiczną preparatu, ryzyko reakcji immunologicznych przy

przeszczepach allogenicznych oraz na standaryzację protokołów wytwarzania MSC.

## **Wnioski**

Zaprezentowany cykl publikacji potwierdza, że zarówno warunki hodowli w hipoksji, jak i zastosowanie statyn w odpowiednich stężeniach mogą pozytywnie wpływać na proliferację i funkcje ludzkich mezenchymalnych komórek macierzystych in vitro. Tak zoptymalizowane MSC, w połączeniu z odpowiednimi technikami podania (np. za pomocą klejów tkankowych czy hydrożeli), mają szansę usprawnić gojenie ran w modelu klinicznym, szczególnie w tak wymagającej grupie pacjentów jak chorzy z zespołem stopy cukrzycowej.

W warunkach klinicznych terapia komórkowa z użyciem allogenicznych ADSC w stopie cukrzycowej prowadziła do przyspieszenia procesu gojenia ran i wyższej częstości całkowitego wygojenia, bez istotnych zdarzeń niepożądanych. Analizy proteomiczne oraz obserwacje żywotności komórek w ranie potwierdzają, że MSC nie tylko przetrwały w trudnym środowisku miejscowym, ale też aktywnie modulowały lokalną odpowiedź zapalną i stymulowały procesy naprawcze poprzez zwiększoną ekspresję białek proangiogennych oraz immunomodulujących.

Praca pogładowa stanowi wskazówkę dla przyszłych badaczy, pokazując z jednej strony potencjał MSC w regeneracji tkanek przy różnego rodzaju owrzodzeniach, a z drugiej wyzwania związane z przejściem od badań laboratoryjnych do rutynowych zastosowań klinicznych. Istotne obszary rozwoju to: precyzyjne określenie optymalnych dawek i częstotliwości podań, wdrożenie spersonalizowanych protokołów (np. z uwzględnieniem dodatkowych czynników, takich jak wiek pacjenta czy stan zaawansowania owrzodzenia), a także dalsze doskonalenie metod modyfikacji MSC w celu zwiększenia ich przeżywalności w tkankach.

Podsumowując, przedstawiony cykl publikacji dostarcza spójnego obrazu wielopłaszczyznowych badań, wykazujących korzystne działanie MSC w środowisku hipoksji i pod wpływem statyn oraz potwierdzających skuteczność i bezpieczeństwo zastosowania terapii komórkowej w leczeniu przewlekłych ran u pacjentów z cukrzycą. Wyniki te mogą stać się podstawą dalszego rozwoju medycyny regeneracyjnej i wdrażania innowacyjnych rozwiązań w codziennej praktyce klinicznej.

### 3. Streszczenie w języku angielskim

*Title: The influence of selected parameters on the metabolism of human mesenchymal stem cells and their regenerative potential in wound therapy*

#### **Introduction**

The objective of this doctoral dissertation is a comprehensive analysis of the impact of selected in vitro culture conditions on the metabolism of human mesenchymal stem cells (MSC), along with the evaluation of their therapeutic efficacy in a clinical model of chronic wounds, exemplified by diabetic foot ulcer (DFU). Given the dynamic advancement in regenerative medicine and the necessity of developing effective methods to enhance tissue repair processes, understanding the mechanisms underpinning the beneficial effects of MSC on tissue regeneration becomes critical.

MSC are characterized by self-renewal capacity, multilineage differentiation potential (including osteoblasts, chondrocytes, and adipocytes), and effective paracrine activity manifested by modulation of inflammatory responses and stimulation of angiogenesis. Existing research suggests potential applications for statins, pleiotropic medication, which may enhance MSC functions under specific environmental conditions. Additionally, oxygen concentration in the culture environment (normoxia versus hypoxia) is an essential factor influencing MSC metabolism, proliferation, and differentiation.

Understanding the mechanisms that lead to improved wound healing under conditions of increased metabolic demand and impaired perfusion (as observed in diabetes) is crucial for the implementation of innovative therapies in everyday clinical practice. This series of publications contributes to expanding knowledge on the effects of statins and hypoxia on MSC, as well as clinical protocols for MSC application in chronic wounds.

To date of publication, no study has previously assessed the combined influence of statins and reduced oxygen concentration in MSC cultures. Study no. 1 investigated the effects of atorvastatin and rosuvastatin on MSC under varying oxygen conditions, also considering donor age. Study no. 2 conducted in vivo experiments: adipose-derived mesenchymal stem cells (ADSC) were applied to diabetic foot ulcer to verify the efficacy of cellular therapy in treating chronic wounds. Study no. 3 reviewed literature

and previous study results to assist future researchers in optimizing the design of cellular therapies for wounds, such as DFU and other chronic wounds.

## **Materials and methods**

In the initial stage of the study, human mesenchymal stem cells derived from bone marrow (BM-MSC) were obtained from 12 patients after receiving their written consent. Ethical approval was granted by the Local Bioethics Committee. The study was conducted at the Department of Immunology, Transplantology and Internal Diseases of the Medical University of Warsaw. Standard phenotypic characterization of cells (expression of CD73, CD90, CD105 antigens, and absence of markers such as CD45, CD34) and differentiation assays (osteogenic, chondrogenic, and adipogenic lineages) were performed.

Two culture environments were tested to evaluate the impact of oxygen conditions. The first group involved normoxic conditions (21% O<sub>2</sub>, standard laboratory conditions), while the second employed hypoxic conditions (2% O<sub>2</sub>, reflecting cellular hypoxia). Cells were incubated with atorvastatin at 0.05 µM (close to physiological therapeutic concentrations in patients) and rosuvastatin at 0.04 µM. Cytotoxicity tests were additionally performed using lower concentrations and a higher concentration (1 µM). Proliferation was assessed by BrdU (5-bromo-2'-deoxyuridine) assay, and cytotoxicity was evaluated by measuring LDH (lactate dehydrogenase) activity in the culture medium.

Study no. 2 summarized a clinical trial involving patients with diabetic foot ulcers. Ethical approval (KB/128/2019 24.06.2019 and KB/3/A2021 18.01.2021) was obtained, and the study was registered on ClinicalTrials.gov (NCT03865394). The study group comprised 47 patients divided into two subgroups: patients receiving ADSC embedded in fibrin carriers and those receiving fibrin glue alone (control group). ADSC isolated from adipose tissue of healthy donors were applied to wound surfaces, and healing progression and the time to 50% ulcer size reduction were monitored. Additionally, wound samples underwent proteomic analysis and assessment for donor-derived DNA to determine cell survival duration post-application.

Study no. 3 consisted of a literature review summarizing:

- MSC mechanisms of action (paracrine effects, immunomodulation, etc.),
- results of key preclinical and clinical MSC studies,
- potential therapeutic protocol modifications such as MSC preconditioning, genetic modifications, or scaffolds to enhance MSC survival and integration within wounds,
- limitations and perspectives of MSC therapy development for chronic wounds, particularly in diabetes.

The review also emphasized MSC application safety concerns, particularly immunogenicity risks under allogeneic conditions and possible inflammatory reactions.

## **Results**

Observations from study no. 1 identified optimal preconditioning conditions using statins and variable oxygen levels for effective MSC performance. Key findings included:

- Physiological concentrations of atorvastatin significantly increased MSC proliferation under both normoxic and hypoxic conditions without substantial cytotoxicity;
- Rosuvastatin stimulated proliferation in hypoxic conditions without significant toxic effects, while slight dose-dependent cytotoxicity increases were observed under normoxia;
- Hypoxia alone enhanced MSC proliferative capacity, suggesting cells better adapt to low oxygen environments typical in ischemic tissues;
- Donor age did not significantly affect MSC response to statins, although generally older donors showed lower MSC proliferative activity.

Study no. 2 observed the following results from long-term patient monitoring with DFU:

- Faster wound size reduction in the ADSC-treated group compared to controls, demonstrated by shorter times to 50% ulcer reduction and higher complete healing rates;
- Proteomic analysis indicated increased expression of regenerative and angiogenesis-related proteins (GAPDH, CAT, etc);



- Donor DNA detection at wound sites persisted for approximately 2-3 weeks post-application, suggesting adequate MSC survival duration for initiating beneficial wound healing effects;
- No serious adverse events directly linked to MSC administration occurred, confirming clinical safety.

The findings of studies 1 and 2 significantly contributed to the review paper (study no. 3), summarizing previous observations and highlighting future research directions:

- MSC wound healing mechanisms involve immunomodulation, angiogenesis stimulation (e.g., VEGF secretion), and direct tissue regeneration support;
- Culture parameters (oxygen concentration, pleiotropic drugs, donor age) optimize MSC therapeutic potential;
- Therapeutic enhancement strategies include genetic modifications, MSC activation by stimuli (hypoxia, growth factors), and cell-scaffold integration (hydrogels or tissue constructs);
- Safety issues and standardization: microbiological purity, immunological reaction risks in allogeneic transplantation, and standardized MSC production protocols.

## **Conclusions**

This series of publications demonstrates that hypoxic conditions and appropriate statin concentrations positively impact human MSC proliferation and functionality *in vitro*. Optimized MSC, combined with suitable administration techniques (tissue adhesives, hydrogels), can improve wound healing in clinical models, particularly challenging diabetic foot ulcer patients.

Clinically, allogeneic ADSC therapy accelerated diabetic foot wound healing, increasing complete recovery frequency without significant adverse events. Proteomic analysis and cellular survival observations confirm MSC survival in challenging local environments, actively modulating inflammatory responses and stimulating repair via proangiogenic and immunomodulatory protein expression.

The review provides guidance for future researchers, highlighting MSC regenerative potential and challenges transitioning from laboratory research to clinical practice. Essential development areas include precise dosage and administration protocols,

personalized approaches (considering patient age or ulcer severity), and MSC modification strategies enhancing tissue survival.

In summary, this publication series offers comprehensive evidence of MSC benefits under hypoxia and statins, confirming cellular therapy's efficacy and safety in treating chronic diabetic wounds, laying the foundation for regenerative medicine advancements and innovative clinical applications.

#### **4. Wstęp uzasadniający połączenie wskazanych publikacji w jeden cykl oraz komentujący osiągnięcia naukowe na tle dotychczasowego stanu wiedzy**

Cukrzyca stanowi jedno z najpoważniejszych wyzwań współczesnej medycyny, ze względu na stały wzrost częstości jej występowania oraz indukcję licznych, trudnych do leczenia powikłań. Według danych International Diabetes Federation (IDF) na świecie żyje już ponad 530 milionów osób z cukrzycą, a liczba ta może wzrosnąć do blisko 800 milionów w połowie bieżącego stulecia (1). Cukrzyca definiowana jest jako choroba metaboliczna, której głównym kryterium rozpoznania jest przewlekła hiperglikemia wynikająca z upośledzonego wydzielania bądź działania insuliny. Najczęstsze postaci cukrzycy to cukrzyca typu 1 (uwarunkowana autoimmunologicznie), cukrzyca typu 2 (związana z insulinoopornością i względnym niedoborem insuliny) oraz cukrzyca ciężarnych. W dobie nowoczesnych metod diagnostycznych zwiększa się także częstość rozpoznawania innych podtypów cukrzycy, np. cukrzycy monogenowej. Dynamiczny wzrost częstości występowania cukrzycy w populacji świata skutkuje zwiększaniem się liczby chorych z przewlekłymi powikłaniami naczyniowymi, co przekłada się na istotne koszty społeczne i zdrowotne. Długo utrzymująca się hiperglikemia prowadzi do rozwoju uszkodzeń śródbłonna, powstania zaawansowanych produktów glikacji (AGE), stresu oksydacyjnego oraz przewlekłego stanu zapalnego. Wszystkie te procesy mają kluczowy wpływ na patogenezę powikłań w obrębie dużych naczyń tętniczych (makroangiopatia) i małych naczyń (mikroangiopatia). Konsekwencjami są m.in. przyspieszona miażdżycy, retinopatia cukrzycowa, nefropatia cukrzycowa, a także polineuropatia cukrzycowa.

Jednym z najpoważniejszych i zarazem najczęstszych powikłań mikro- i makroangiopatycznych jest zespół stopy cukrzycowej, będący główną przyczyną amputacji niezwiązanych z urazami oraz najczęstszą przyczyną hospitalizacji pacjentów chorujących na cukrzycę (2). Obecnie według najnowszej definicji Międzynarodowej Grupy Roboczej ds. Stopy Cukrzycowej (IWGDF) rozszerzono pojęcie stopy cukrzycowej do tzw. Cukrzycowej choroby stóp, która obejmuje zarówno klasyczne postaci (do niedawna nazywane zespołem stopy cukrzycowej) oraz stany, które do niej doprowadzają (neuropatia oraz stany miażdżycowe tętnic kończyn dolnych). W przypadku możliwości dysponowania wystarczającymi środkami wczesnej prewencji czynników etiologicznych cukrzycowej choroby stóp, prawdopodobnie nie było potrzeby

poszukiwania zaawansowanych metod terapeutycznych do przeciwdziałania tym czynnikom.

Głównym czynnikiem etiologicznym powstawania i utrzymywania się trudno gojących się ran w cukrzycy jest neuropatia cukrzycowa. Powstaje ona w wyniku długotrwałego działania hiperglikemii na drobne naczynia oraz na same włókna nerwowe, co prowadzi do ich niedotlenienia i uszkodzenia. Proces ten obejmuje zarówno włókna czuciowe, ruchowe, jak i autonomiczne. W efekcie dochodzi do zaburzeń czucia bólu, temperatury czy wibracji, co może sprzyjać tworzeniu się niezauważonych otarć, pęknięć naskórka i drobnych urazów. Ponadto dysfunkcja autonomiczna prowadzi do suchości skóry oraz zaburzeń troficznych w obrębie kończyn dolnych. Pacjent często nie odczuwa bólu, a więc nie chroni stopy przed mikrourazami. Brak mechanizmu ostrzegawczego powoduje progresję niewielkich uszkodzeń do głębokich owrzodzeń. Dodatkowo występują zaburzenia ukrwienia stopy, które wynikają z zaawansowanej miażdżycy tętnic obwodowych, zwłaszcza u pacjentów z wieloletnią cukrzycą typu 2. Uszkodzony śródbłonek naczyniowy i nasilona proliferacja komórek mięśni gładkich w ścianie tętnic prowadzą do zwężenia światła naczyń oraz ograniczenia perfuzji tkanek dystalnych. Niska podaż tlenu i substancji odżywczych przekłada się na zaburzenia procesów gojenia się ran (3). Infekcje w warunkach niedotlenienia mają większą skłonność do szybkiego rozprzestrzeniania się w głąb tkanek, prowadząc często do rozległych stanów zapalnych, a nawet posocznicy. Szczegółowy opis procesów patofizjologicznych wpływających na upośledzenie gojenia się ran w przebiegu cukrzycy zawarty jest w pracy nr 3 składającej się na ten cykl publikacji, dlatego w tej części nakreślony został jedynie zarys tych procesów.

Jak wspomniano powyżej, jednym z najcięższych powikłań wieloletniej cukrzycy jest wystąpienie owrzodzenia definiowanego jako stopa cukrzycowa, co niejednokrotnie kończy się amputacją kończyny. Dodać zdanie nt. innych rodzajów ran Owrzodzenia kończyn dolnych dotyczą nawet jednej trzeciej pacjentów w ciągu ich życia, prowadząc do znacznej zachorowalności, charakteryzującej się trudnym przebiegiem leczenia i wysokim wskaźnikiem nawrotów (4).

Z epidemiologicznego punktu widzenia głównymi czynnikami ryzyka rozwoju ZSC są czas trwania cukrzycy, wyrównanie metaboliczne choroby, współistniejąca przewlekła choroba nerek, nadciśnienie tętnicze, hiperlipidemia oraz palenie papierosów. Nie bez znaczenia pozostaje czynnik socjoekonomiczny, a zwłaszcza utrudniony dostęp do nowoczesnych metod diagnostycznych, brak odpowiedniej edukacji pacjenta

w zakresie pielęgnacji stóp czy zbyt późne wdrożenie leczenia. W codziennej praktyce klinicznej w leczeniu owrzodzeń w przebiegu ZSC stosuje się przede wszystkim metody mające na celu redukcję obciążenia uszkodzonej stopy (odciążanie), odpowiednią kontrolę zakażenia (antybiotykoterapię celowaną, interwencje chirurgiczne), poprawę ukrwienia (leczenie rewaskularyzacyjne), a także skuteczną kontrolę glikemii. Mimo tych wysiłków, wiele ran jest trudnych do wyleczenia, zwłaszcza w zaawansowanych stadiach niedokrwienia i/lub neuropatii (5, 6). Stąd od lat poszukuje się nowych metod terapeutycznych umożliwiających poprawę ukrwienia i przyspieszenie procesów regeneracyjnych. Czynniki predysponującymi do szybkiego pogarszania się stanu miejscowego rany u pacjentów z zespołem stopy cukrzycowej są wspomniana neuropatia czuciowa, która sprawia, że chory rzadko zgłasza dolegliwości bólowe, oraz utrudniony przepływ krwi w tętnicach obwodowych, który uniemożliwia szybką odpowiedź immunologiczną i właściwą dystrybucję leków do tkanek. Ze względu na niewystarczającą skuteczność obecnych metod leczenia trudno gojących się ran w przebiegu cukrzycy, intensywnie prowadzi się badania nad nowymi strategiami terapeutycznymi. Jednym z obszarów szczególnie zainteresowania jest zastosowanie mezenchymalnych komórek macierzystych, które ze względu na swój potencjał regeneracyjny i immunomodulacyjny mogą stanowić ważne wsparcie w terapii ran przewlekłych. (7)

MSC to populacja komórek multipotencjalnych, które można izolować z wielu tkanek organizmu, w tym szpiku kostnego, tkanki tłuszczowej, krwi pępowinowej czy galarety Whartona. (8) W warunkach *in vitro* wykazują one zdolność różnicowania się m.in. w kierunku osteocytów, chondrocytów i adipocytów, a także wyróżniają się wydzielaniem licznych czynników wzrostu (np. VEGF, FGF czy HGF), cytokin immunomodulujących i białek o działaniu przeciwzapalnym. (9) Z tego powodu MSC były już testowane w wielu modelach regeneracji tkanek, w tym w gojeniu ran skórnych czy regeneracji mięśnia sercowego. (10)

Statyny, to leki z grupy inhibitorów reduktazy 3-hydroksy-3-metyloglutarylokoenzymu A (HMG-CoA), powszechnie stosowane w celu obniżania poziomu cholesterolu frakcji LDL i redukcji ryzyka sercowo-naczyniowego. W ostatnich latach wykazano jednak szereg efektów pleiotropowych tych leków, co oznacza, że ich działanie wykracza poza redukcję stężenia cholesterolu. Okazuje się, że statyny mogą wpływać m.in. na proces zapalny, funkcję śródbłonna, czy angiogenezę. W kontekście gojenia ran interesujące są doniesienia, że statyny mogą modulować aktywność MSC - zarówno pobudzać proliferację tych komórek, jak i zwiększać ich potencjał parakryny. (10) W hodowli

komórek zwierzęcych wykazano, że statyny hamują w MSC szlak apoptozy indukowanej hipoksją (11) i nasilają proces autofagii (12). W badaniach na modelu świńskim podanie atorwastatyny skutkowało zwiększoną skutecznością terapii z użyciem MSC po zawale serca (13).

Hipoksja, czyli obniżone stężenie tlenu w środowisku, stanowi jeden z istotnych czynników patogenetycznych w procesie rozwoju stopy cukrzycowej. Niedotlenienie tkanek pogarsza rokowanie co do wygojenia ran, zwłaszcza w obecności infekcji. Z drugiej strony, istnieją doniesienia naukowe sugerujące, że krótkotrwała ekspozycja MSC na warunki niedotlenienia (czy też inne bodźce stresowe) może wzmacniać ich potencjał regeneracyjny oraz wydzielanie cytokin i czynników wzrostu, zjawisko to nazywane jest prekondycjonowaniem. Powszechnie wiadomo, że stężenie tlenu znacząco wpływa na zachowanie komórek. Analiza wykazała, że ponad 440 genów MSC było w różny sposób regulowanych pod wpływem niedotlenienia (0,5% O<sub>2</sub> przez 24 h) w porównaniu do standardowych warunków hodowli (21% O<sub>2</sub>) (14). Wpływ hipoksji na aktywność MSC jest szczególnie istotny, jeśli chodzi o potencjał terapeutyczny leczenia powikłań wielu chorób, takich jak ostry zespół wieńcowy. Wpływ hipoksji badano również u pacjentów z zespołem stopy cukrzycowej, gdzie powtarzalne zaciski mankiету na przedramieniu wywoływały przyspieszenie procesu gojenia się rany na przeciwległej kończynie dolnej w porównaniu do grupy pacjentów, u których nie stosowano powyższego sposobu interwencji (15).

W pracy nr 1 niniejszego cyklu wykazano, że atorwastatyna i rosuwastatyna mogą w warunkach *in vitro* wspomagać proliferację mezenchymalnych komórek macierzystych i jednocześnie nie wywoływać istotnych efektów cytotoksycznych, zwłaszcza w stężeniach zbliżonych do tych osiąganych w surowicy pacjentów leczonych standardowymi dawkami. Szczególnie interesujący jest fakt, iż wpływ statyn na MSC może być zależny od poziomu tlenu w środowisku hodowli (normoksja vs. hipoksja) oraz od wieku dawcy komórek. Zależności te mają kluczowe znaczenie przy projektowaniu potencjalnych zastosowań klinicznych.

Połączenie statyn i kontrolowanej hipoksji w kontekście prekondycjonowania MSC wydaje się szczególnie obiecujące, gdyż oba czynniki - choć różnymi szlakami - mogą poprawiać zdolności regeneracyjne tych komórek. Statyny mogą wspomagać przeżycie MSC w niekorzystnych warunkach tlenowych, natomiast hipoksja w sposób naturalny „przygotowuje” komórki do funkcjonowania w niedotlenionym obszarze rany, jednocześnie stymulując wydzielanie czynników wzrostu. Mechanizmy te wymagają

dalszego zbadania *in vitro* i *in vivo*, ale już obecne dane opisane w pracach będących częścią tej rozprawy wskazują na ich realny potencjał kliniczny.

Korzystny wpływ MSC na gojenie ran w cukrzycy jest szeroko omawiany w literaturze. W pracy nr 2 niniejszej rozprawy wykazano, że ADSC sprzyjają gojeniu się ran w stopie cukrzycowej poprzez zwiększenie angiogenezy i modulację stanu zapalnego. Z kolei w pracy nr 3 zwrócono uwagę na wielokierunkowe mechanizmy działania MSC - w tym na zdolność do wydzielania czynników wzrostu, cytokin immunomodulujących i proteolitycznych enzymów uczestniczących w przebudowie macierzy pozakomórkowej. Łączenie tych komórek z dodatkowymi metodami wspomagającymi, takimi jak zastosowanie statyn, czynników wzrostu czy warunków hipoksji, może prowadzić do jeszcze bardziej efektywnej terapii.

Równocześnie warto podkreślić, że w dotychczasowych badaniach klinicznych z udziałem MSC, część pacjentów z cukrzycą odniosła znaczącą poprawę w zakresie gojenia się ran stopy, co przekładało się na zmniejszenie ryzyka amputacji oraz poprawę jakości życia. Z tego powodu opracowanie protokołów klinicznych optymalizujących wykorzystanie MSC u chorych z zaawansowanym zespołem stopy cukrzycowej wydaje się obiecującym kierunkiem badań.

Przedstawione w cyklu trzy publikacje koncentrują się na powiązanych aspektach:

- praca nr 1 odnosi się do badania efektów statyn w zależności od stężenia tlenu oraz wieku dawcy MSC w warunkach *in vitro*;
- prace nr 2 i 3 omawiają potencjał MSC w terapii przewlekłych ran, z naciskiem na mechanizmy molekularne, w tym udział cytokin i czynników wzrostu.

Wspólnym mianownikiem tych prac jest dążenie do optymalizacji terapii komórkowych w warunkach, jakie spotykamy u pacjentów z cukrzycą i powikłaniami naczyniowymi, w tym w zespole stopy cukrzycowej. W każdym z artykułów analizowano potencjalne sposoby wzmacniania właściwości MSC (statyny, hipoksja, protokoły hodowli), co ostatecznie ma służyć poprawie skuteczności klinicznej. Takie wielopłaszczyznowe spojrzenie, od badań podstawowych po wskazania kliniczne, czyni prace komplementarnymi i uzasadnia ich włączenie w jeden cykl rozprawy doktorskiej.

## 5. Założenia i cel pracy

Głównym celem rozprawy doktorskiej jest zbadanie wpływu statyn oraz hipoksji na wybrane parametry metaboliczne mezenchymalnych komórek macierzystych (MSC) oraz ocena potencjału terapeutycznego tych komórek, zwłaszcza w kontekście leczenia trudno gojących się ran w przebiegu cukrzycy (w tym zespołu stopy cukrzycowej).

- **Prekondycjonowanie z użyciem statyn oraz hipoksji może wpływać korzystnie na proliferację oraz przeżywalność MSC, a tym samym potencjalnie wspierać ich zastosowanie w regeneracyjnych terapiach ukierunkowanych na uszkodzenia niedokrwienne.**
- **ADSC są zdolne do przyspieszania procesu gojenia ran w zespole stopy cukrzycowej.**

Celem rozprawy było:

1. Określenie wpływu wykorzystania statyn w procesie prekondycjonowania na aktywność i cytotoksyczność ludzkich MSC w warunkach normoksji i hipoksji, z uwzględnieniem potencjalnych różnic związanych z wiekiem dawcy.
2. Ocena klinicznej skuteczności oraz bezpieczeństwa podania allogenicznych ADSC w leczeniu przewlekłych owrzodzeń stopy cukrzycowej, w tym w kontekście zmiany rozmiaru rany i częstości całkowitego wygojenia.
3. Analiza molekularnych podstaw działania MSC na proces gojenia ran, uwzględniająca m.in. badania proteomiczne łożyska rany i ocenę czasu przeżycia komórek dawcy w miejscu owrzodzenia, w celu lepszego zrozumienia mechanizmów regeneracyjnych.



## 6. Kopie opublikowanych prac

### 6.1. The influence of oxygen deprivation and donor age on the effect of statins on human mesenchymal stromal cells

Tissue and Cell 67 (2020) 101427



Contents lists available at ScienceDirect

Tissue and Cell

journal homepage: [www.elsevier.com/locate/tice](http://www.elsevier.com/locate/tice)



## The influence of oxygen deprivation and donor age on the effect of statins on human mesenchymal stromal cells

Damian Sienko<sup>a,1</sup>, Dominika Klimczak-Tomaniak<sup>a,b,1</sup>, Agnieszka Kulesza<sup>a</sup>, Helena Symonides<sup>a</sup>, Marek Kuch<sup>b</sup>, Leszek Paczek<sup>a,c</sup>, Anna Burdzinska<sup>a,\*</sup>

<sup>a</sup> Department of Immunology, Transplantation and Internal Diseases, Medical University of Warsaw, Nowogrodzka 59, 02-006 Warsaw, Poland

<sup>b</sup> Department of Cardiology, Hypertension and Internal Medicine, Medical University of Warsaw, Kondratowicza 8, 03-242, Warsaw, Poland

<sup>c</sup> Department of Bioinformatics, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawłńskiego 5A, 02-106 Warsaw, Poland

#### ARTICLE INFO

##### Keywords:

Atorvastatin

Rosuvastatin

Mesenchymal stromal cells

Hypoxia

#### ABSTRACT

To date, no study evaluated the effect of oxygen deprivation together with statins pretreatment on human mesenchymal stromal cells (MSCs). The aim of our study was to establish the influence of atorvastatin and rosuvastatin on MSC proliferation and cytotoxicity in different oxygenic conditions. Human MSCs isolated from the bone marrow ( $n = 12$ ) were incubated with statins. The proliferation rate and cytotoxic effect were evaluated in normoxic (21 %O<sub>2</sub>) and hypoxic (2%O<sub>2</sub>) conditions, also in relation to donor age. The treatment with atorvastatin was associated with significantly higher proliferation rate compared to control, both in hypoxic (19 % median increase) and normoxic conditions (20 %),  $p = 0.02$  and  $p = 0.04$ , respectively. Atorvastatin had no significant cytotoxic effect on MSCs. Treatment with rosuvastatin in hypoxia resulted in significantly higher proliferation rate (15 %,  $p = 0.02$ ) comparing to control with no significant cytotoxicity. In atmospheric oxygen concentration, rosuvastatin was associated with no significant change in proliferation and higher cytotoxicity compared to untreated control ( $p = 0.042$  and  $p = 0.015$ , for 0.04  $\mu$ M and 1  $\mu$ M solutions respectively). There were no differences in the effect of statins on MSC from young donors vs. aged donors. These results suggest that statins could support MSC-based therapy of acute myocardial infarction.

#### 1. Introduction

Mesenchymal stromal cells (MSC) constitute a population primarily residing in the bone marrow where they support the process of haematopoiesis. Apart from bone marrow, they can also be isolated from a variety of human tissues, including adipose tissue, umbilical cord blood, and Wharton's jelly (Secunda et al., 2015). MSCs can be easily expanded *in vitro*, are able differentiate into several cell types and therefore are perceived as a material for the repair or rejuvenation of diseased and ageing tissues (Hwang et al., 2009). Myocardial infarction is a leading cause of morbidity and mortality worldwide. Results of many preclinical studies indicate that MSC transplantation can improve left ventricular ejection fraction in animals with induced ischemic heart disease (van der Spoel et al., 2011). It seems that exact mechanism of MSC action after intramyocardial injection is still to be clarified, but meta-analyses of results obtained during controlled clinical trials in patients with acute myocardial infarction (AMI) (Lalu et al., 2018) suggest that MSC

transplantation can have beneficial functional effect, which justify further efforts to investigate the potential for MSC in this group of patients.

It is known that different drugs can affect activity of both endogenous and transplanted MSC. Statins seem to be of particular interest in the treatment of patients after myocardial infarction and one of the most widely prescribed medicaments in cases of ischemic heart disease. These pharmaceuticals have gained more interest in recent decades due to the pleiotropic abilities they present. Interestingly, statins have been shown to have a beneficial impact on transplanted MSC activity in animal models with AMI (Yang et al., 2009; Dai et al., 2015). Therefore, it seems important to evaluate the influence of statins on the proliferative potential of human MSC. Previous reports regarding the *in vitro* effect of statins on MSC are not consistent. Zhang et al. (2012) demonstrated, that in conditions which induce apoptosis (hypoxia with serum deprivation) statins have protective effect on MSCs by inducing autophagy (instead of apoptosis). On the other hand, many other reports indicate that statins

\* Corresponding author.

E-mail address: [anna.burdzinska@wum.edu.pl](mailto:anna.burdzinska@wum.edu.pl) (A. Burdzinska).

<sup>1</sup> Equally contributing authors.

<https://doi.org/10.1016/j.tice.2020.101427>

Received 12 May 2020; Received in revised form 12 August 2020; Accepted 13 August 2020

Available online 15 August 2020

0040-8166/© 2020 Published by Elsevier Ltd.



impair MSC viability (Baek et al., 2005; Izadpanah et al., 2015; Li et al., 2015; Zanette et al., 2015). However, it is important to state that cytotoxic effect of statins in these studies was observed mainly if drugs were used in concentrations many times higher than those found in the patient's serum. (Baek et al., 2005)

Another issue is the effect of oxygen conditions on MSC activity. The microarray analysis revealed that the expression of over 440 genes was changed under the influence of severe hypoxia (0.5% O<sub>2</sub> for 24 h) in bone marrow MSCs in comparison to standard culture conditions (21 % O<sub>2</sub>) (Hu et al., 2014). The effect of hypoxia on MSC activity appears to be particularly important in the potential treatment of AMI. The impact of oxygen conditions on the effect of statins on human MSCs was not reported until now.

Finally, considering MSC transplantation in cases of cardiomyopathies it is reasonable to take into account the age of cell donors. Although there is an increasing amount of data that allogeneic MSCs transfer is safe (Lin et al., 2012), autologous therapy is still chosen in many clinical trials. Moreover, the dogma that allogeneic MSCs are immunologically privileged is currently being questioned by some authors (Berglund et al., 2017). Some animal studies indicate that major histocompatibility complex mismatched MSC transplantation can induce an immune response and graft elimination (Berglund and Schnabel, 2017). Therefore, the question about the impact of aging on cell features is still valid, especially that previous reports indicate that donor's age affects the proliferation capacity of human MSCs isolated from bone marrow (Li et al., 2014a).

Taken together, there were several objectives of the present study. First, to evaluate the effect of statins (atorvastatin and rosuvastatin) used in concentrations which are found in patients' serum on human mesenchymal stromal cells. Second, to assess what is the influence of different oxygen conditions (atmospheric oxygen vs 2% oxygen) on the effect of statins on human MSCs. And third, to determine if donor age influences the effect of statins on MSC proliferation rate.

## 2. Material and methods

### 2.1. Material and donors

Bone marrow samples were obtained from 12 patients after receiving their written consent. The procedure was approved by the Local Bioethics Committee (approval numbers: KB/130/2013 and KB/115/2016). The bone marrow was collected during standard orthopaedic surgeries which required opening of bone marrow cavity. All sample donors were male; the mean age was 50 years. For some analyses samples were divided depending on donor age for two groups: young (Y-MSC, N = 6) – cells from donors younger than 40 years old (the mean age in this group was 33 ± 6.8) and aged (A-MSC, N = 6) – cells from donors older than 40 years (the mean age in this group was 67.8 ± 6).

### 2.2. Cell isolation and expansion

Bone marrow, after mechanical dissociation (pushing through a syringe and, if possible, 26 G needle), was washed with PBS and centrifuged for 5 min at 300g. The samples were then expanded in growth medium composed of DMEM-LG (Dulbecco's modified Eagle's Medium with low glucose; LONZA) supplemented with fetal calf serum (FCS; 10 %; LONZA) and antibiotic-antimycotic (AA) solution (Penicillin-streptomycin-amphotericin B; 1.5 %; Invitrogen), seeded on BD Primaria™ culture dishes (Becton Dickinson) and incubated under standard cell culture conditions (37 °C, 5% CO<sub>2</sub>, 95 % humidity, and atmospheric oxygen concentration). After 2 days growth medium (GM) was replenished and after another 2 days non-adherent cells was removed and first fibroblast-like adherent cells could be observed. After 7–10 days (depending on the primarily attached cell number), when the colonies were well formed the cells were passaged. Subsequent passages were performed when cells were reaching about 80 % of confluence.

After the 3rd passage the cells were frozen in liquid nitrogen. The experiments were performed after thawing, on cells between 4–6 passage. The cells from different donors were always cultured separately. In each set of experiments one population from A-MSC group and one population from Y-MSC group were analyzed in parallel.

### 2.3. MSCs identification

Isolated cells displayed fibroblastic morphology and created colonies when seeded in a very low density. The ability to multilineage differentiation and the expression of surface antigens were analysed after the 3rd or the 4th passage.

#### 2.3.1. Osteogenic differentiation

The efficacy of osteogenic differentiation was evaluated with two methods – the visualization of calcium deposits (Alizarin red staining) and measuring the activity of alkaline phosphatase (AP) using semi-quantitative colorimetric method. The cells were seeded on 12-well plates (4 wells per donor). When reached 70 % of confluence, the medium on 2 wells were changed into differentiating medium containing 0.1 μM dexamethasone, 10 mM β-glycerophosphate, 50 μM AA-2P (ascorbic acid 2-phosphate) in standard GM with 10 % FBS (all the supplements were purchased from Sigma-Aldrich), while another 2 wells remained negative control (standard GM with 10 % FBS). Culture media were changed every 3 days. After 2 weeks of culturing cells on one control and one differentiating well were fixed for 15 min with 4% formaldehyde and stained with Alizarin Red (Sigma-Aldrich) in order to visualize calcium deposits. On the other control and differentiating well the cells were treated with 0.2 % Triton X-100 in PBS for 20 min. The lysates, after centrifugation, were combined with reaction substrate (The Alkaline Phosphatase Liquid Substrate assay, Sigma-Aldrich, P7998) and incubated for up to 30 min in RT and read at 405 nm according to the manufacturer instruction.

#### 2.3.2. Adipogenic differentiation

The efficacy of adipogenic differentiation was evaluated by visualization of lipid droplets using Oil Red O staining. The cells were seeded on 12-well plates (2 wells per donor). When reached 90–100 % of confluence, one dish was started being treated with differentiation medium containing 0.5 μM IBMX (isobutyl methyl xanthine), 1 μM dexamethasone, 10 μg/mL insulin, 60 μM indomethacin in standard GM with 10 % FBS (all the supplements were purchased from Sigma-Aldrich), while the second dish remained negative control (standard GM with 10 % FBS). Culture media were changed every 3 days. After 3 weeks cells were fixed for 45 min with 10 % formaldehyde and then for 5 min with 60 % isopropanol and stained with Oil Red O (Sigma-Aldrich) in order to visualize lipid droplets.

#### 2.3.3. Chondrogenic differentiation

To assess the ability to chondrogenic differentiation, three-dimensional pellet culture was performed. Unsuspended cell pellets were cultured for two-three weeks in chondrogenic medium composed of DMEM high glucose medium supplemented with 0.5 % FBS, 1% (v/v) ITS (insulin-transferrin-selenium), 0.1 μM dexamethasone, 100 μM ascorbic acid 2-phosphate, 100 μg/mL sodium pyruvate, and 10 ng/mL recombinant human transforming growth factor-β2 and 1% (v/v) penicillin-streptomycin solution. Medium were changed every 3 days. For histological analysis, pellet was immersed in paraffin, sectioned and stained with haematoxylin-eosin.

#### 2.3.4. Assessment of surface antigens

Phenotype of cells was determined with the use of Human MSCs Analysis Kit (BD Biosciences). Expression of positive (CD73, CD90, CD105, CD44) and negative (CD34, CD45, CD11b, CD19, HLA-DR) cell surface markers were investigated. Cells were detached, rinsed 2 times with PBS and incubated with antibodies following manufacturer's



instructions, then incubated 30 min in the dark. After 3 times rinse, the cells were evaluated using BD FACSCanto II flow cytometer equipped with BD FACSDiva Software.

## 2.4. Cell treatment

### 2.4.1. Reagents and doses

Statins used in this study were: 1. Atorvastatin calcium trihydrate, European Pharmacopoeia (EP) Reference Standard (cat no. Y0001327); 2. Rosuvastatin calcium  $\geq 98\%$  (HPLC) (cat no. SML1264), both from Sigma-Aldrich. The main doses used in this study were:  $0.05\ \mu\text{M}$  ( $27\ \text{ng/mL}$ ) for Atorvastatin and  $0.04\ \mu\text{M}$  ( $37\ \text{ng/mL}$ ) for Rosuvastatin. Both concentrations correspond to the maximum concentration ( $C_{\text{max}}$ ) achieved in patients' serum. Additionally, for cytotoxicity assay, the lower and higher dose was used.

### 2.4.2. Cell proliferation assay

The rate of cell proliferation was determined using a colorimetric 5-bromo-2'-deoxyuridine (BrdU) proliferation ELISA immunoassay (Roche). MSC were seeded into 96-well plates in a density  $4 \times 10^3$  cells per well on 2 plates – one designated to normoxic conditions (atmospheric oxygen concentration) and the second designated to hypoxic conditions (2% oxygen level). For attachment both plates were kept in normoxic conditions. The next day (day 0 of the experiment), the medium was changed and the cells were exposed to statins: atorvastatin –  $0.05\ \mu\text{M}$  or rosuvastatin –  $0.04\ \mu\text{M}$ . Cells cultured in parallel in standard growth medium served as negative control whereas cells cultured in  $5\ \text{ng/mL}$  basic fibroblast growth factor (bFGF) served as positive control to confirm the ability of MSC to respond to mitogenic stimuli. The plates were placed under appropriate oxygen conditions – one in  $21\ \text{O}_2$  and the second in  $2\ \text{O}_2$ . The medium was changed the next day (day 1 of the experiment). At day 2, BrdU was added to each well according to the manufacturer instructions. The incubation was continued for 16–18 hours during which BrdU was incorporated into the DNA of proliferating cells. Further, the immunostaining was performed according to manufacturer instructions. The absorbance was measured at wavelength  $450\ \text{nm}$  (BioTek PowerWave XS). All treatments were performed in triplicates.

### 2.4.3. Cell cytotoxicity assay

To measure the cytotoxic effect of statin on MSCs – a colorimetric assay for quantifying the cytolysis process was used (Cytotoxicity Detection Kit PLUS (LDH) purchased from Roche). The cells were seeded into 96-well plates in a density  $8 \times 10^3$  cells per well on 2 plates. The next day after seeding, the medium was changed and the cells were exposed to statins: atorvastatin –  $0.025\ \mu\text{M}$ ,  $0.05\ \mu\text{M}$  and  $1\ \mu\text{M}$  or rosuvastatin –  $0.02\ \mu\text{M}$ ,  $0.04\ \mu\text{M}$  and  $1\ \mu\text{M}$ . The plates were placed under appropriate oxygen conditions – one in  $21\ \text{O}_2$  and the second in  $2\ \text{O}_2$ . Background control, low control and high control were also included as recommended by manufacturer instruction. The experiment lasted 72 h. The medium was changed once, in the middle of the experiment. At the end, the amount lactate dehydrogenase (LDH) activity was measured in cell culture supernatants. LDH is a stable cytoplasmic enzyme that is rapidly released into the supernatant upon damage of the plasma membrane. The colorimetric analysis was performed using spectrophotometry at wavelength  $490\ \text{nm}$  (BioTek PowerWaveXS). All experiments were done in triplicates. To determine the percentage cytotoxicity of statins on MSCs we calculated absorbance values in the equation suggested by a manufacturer, where:

Cytotoxicity [%] =  $(\text{experimental value} - \text{low control}) / (\text{high control} - \text{low control}) \times 100$

## 2.5. Statistical analysis

All the statistical analyses were conducted using STATISTICA software (StatSoft®). First, the data distribution within groups were

evaluated using Shapiro-Wilk test. The groups of related data with abnormal distribution were compared using Wilcoxon matched-pairs signed-ranks test. Student's t-test was used to compare values groups with confirmed normal distribution. Significance was set at  $p < 0.05$  and graphs are presented as mean  $\pm$  standard error of the mean (SEM).

## 3. Results

### 3.1. MSC identification

Human bone marrow-derived mesenchymal stromal cells (hMSC) were isolated from 12 donors. All MSC populations adhered to the surface of the plastic culture dishes forming fibroblast-like colonies. Flow cytometry analysis of cell-surface antigen profiles revealed that isolated cells possessed mesenchymal stromal cells phenotype. The median percentage of positive cells amounted  $99.3\%$  for CD44,  $99.5\%$  for CD73,  $98.6\%$  for CD90 and  $99.4\%$  for CD105 and  $1.3\%$  for hematopoietic markers (cocktail of CD11b, CD19, CD34, CD45 and HLA-DR). The expression of surface markers did not differ significantly between cells isolated from young and aged donors. In order to confirm the ability of isolated cells to differentiate into osteocytes, chondrocytes and adipocytes, specific staining was successfully performed (Fig. 1). Moreover, osteogenic differentiation was confirmed by measuring alkaline phosphatase. The mean AP activity increased after osteogenic differentiation 4.7-fold comparing to untreated control ( $p = 0.01$ ). There was no significant difference in response to osteogenic differentiation between cells from MSC-Y and MSC-A group.

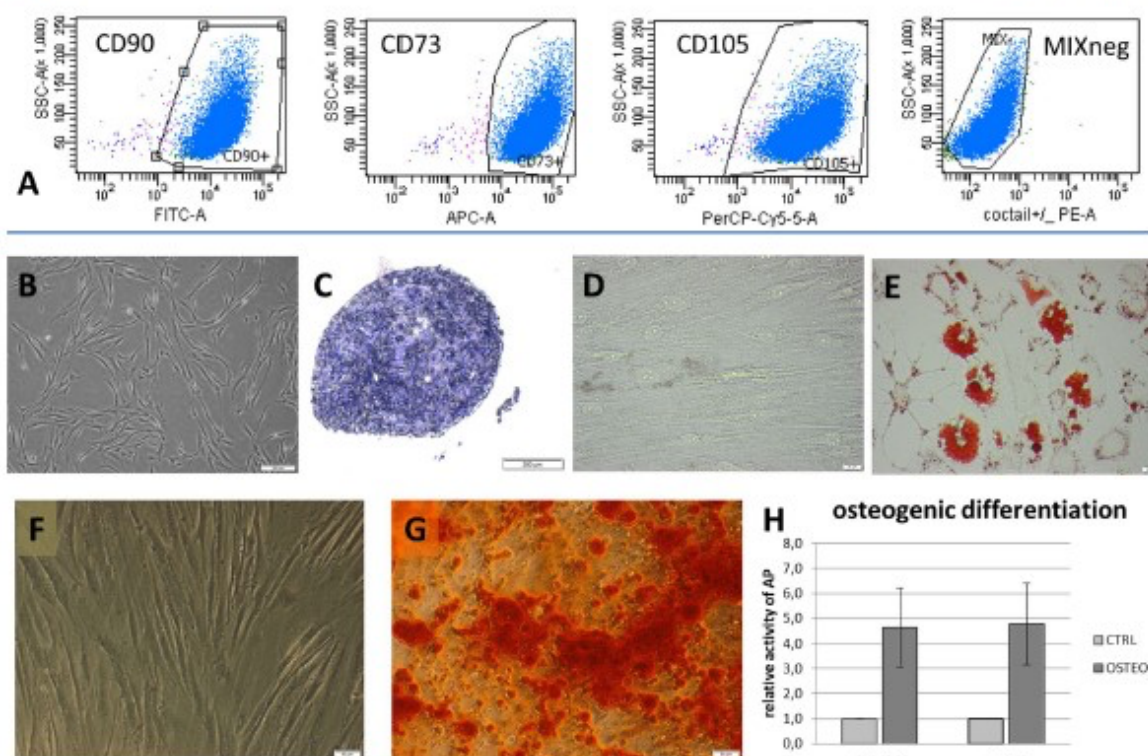
### 3.2. The effect of statins on proliferation rate of hMSCs in different oxygen conditions

The treatment with statins in doses corresponding with the  $C_{\text{max}}$  achieved in patients' serum significantly affected the proliferation rate of human mesenchymal stromal cells. Comparing to untreated cells, the median increase in proliferation after treatment with atorvastatin ( $0.05\ \mu\text{M}$ ) amounted to  $20\%$  in atmospheric oxygen conditions and  $19\%$  in hypoxic conditions ( $p = 0.04$  and  $p = 0.02$ , respectively). Treatment with rosuvastatin ( $0.04\ \mu\text{M}$ ) affected MSC proliferation only in hypoxic conditions (median increase by  $15\%$  in comparison to untreated control,  $p = 0.02$ ), whereas the same treatment in atmospheric oxygen concentration had no effect in BrdU test ( $p = 0.81$ ) (Fig. 2A,B). The analysis of the effect of oxygen conditions on the cell response to statins revealed, that the proliferation rate under influence of rosuvastatin was significantly higher in hypoxia than in "normoxia" (median increase by  $64\%$ ,  $p = 0.028$ ). This effect was not observed in cells treated with atorvastatin (Fig. 2C).

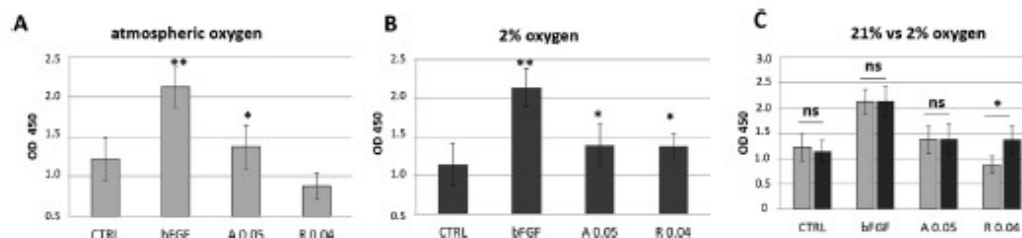
### 3.3. The cytotoxic effect of statins on hMSCs in different oxygen conditions

LDH Colorimetric Assay allowed to assess the effect of statins on the cell membrane damage in hMSCs. For this test statins were used in three different concentrations:  $\frac{1}{2} C_{\text{max}}$ ,  $C_{\text{max}}$  and  $1\ \mu\text{M}$  (which is 20–25 fold higher than  $C_{\text{max}}$  concentration). The results indicate that atorvastatin did not induce significant cell membrane damage in hMSC in any of used doses and regardless of aerobic conditions (Fig. 3A,B). The response to rosuvastatin differed depending on oxygen concentration. In "normoxia" rosuvastatin appeared to significantly increase LDH release in  $C_{\text{max}}$  and  $1\ \mu\text{M}$  in comparison to untreated control ( $p = 0.042$  and  $p = 0.015$ , respectively) (Fig. 3A). This effect was abolished in hypoxic conditions, in which rosuvastatin was not cytotoxic in any concentration (Fig. 3B). Moreover, in hypoxia the amount of released LDH was inversely proportional to the rosuvastatin concentration. These differences were not statistically significant in comparison to the untreated cells, however, cytotoxic effect of rosuvastatin was significantly lower at the  $1\ \mu\text{M}$  dose than in  $C_{\text{max}}$  and  $\frac{1}{2} C_{\text{max}}$ . The comparisons of data from





**Fig. 1.** Morphology and differentiation potential of bone marrow mesenchymal stromal cells (BM-MSCs). A). representative cytometric analysis of surface antigens on BM-MSCs. Negative cocktail (Mix neg) consisted of IgG anti-CD34, CD45, CD11b, CD19 and HLA-DR. B). Untreated BM-MSCs during growth phase; C) BM-MSCs after chondrogenic differentiation, chondropellet stained with Toluidin blue, D, E). Oil Red O staining of hASCs cultured in a standard (D) or adipogenic medium (E) (visible fat droplets stained in red), F, G). Alizarin Red staining of BM-MSCs cultured in a standard (F) or osteogenic medium (G) (visible red calcium deposits), H) The graph representing the relative phosphatase alkaline (AP) activity in control BM-MSCs and after osteogenic differentiation. The results are presented for BM-MSC from younger donors (Y-MSCs) and elder donors (A-MSCs). Scale bars: 200  $\mu$ m (B,C); 50  $\mu$ m (F,G); 20  $\mu$ m (D,E). (This figure should be printed in color).



**Fig. 2.** The effects of statins on the proliferation of bone marrow mesenchymal stromal cells (MSC) in different oxygen conditions (test BrdU). A,B) The graphs present optical density (OD) from BrdU assay in different oxygen conditions. Data analyzed in comparison to control, untreated cells (CTRL). C) The graph present MSC proliferation rate in hypoxia in relation to the analogous treatment in atmospheric oxygen concentration. Data are presented as means and SEM. Statistical analysis was performed by Wilcoxon test.  $p < 0.05$  was assumed to be statistically significant. \* -  $p < 0.05$ , \*\* -  $p < 0.01$ , ns – not significant difference, N = 12; bFGF – basic fibroblasts growth factor, A – Atorvastatin, R – Rosuvastatin, 0.05 and 0.04 – dose in  $\mu$ M (concentration equal to maximal concentration found in human patients' serum).

the same treatments for normoxia and hypoxia revealed no significant differences. Raw data (mean OD490 and SEM) for each group are presented in Suppl. Table 1.

#### 3.4. The effect of statins on hMSCs depending on donor's age

Additionally, the influence of cell donor age on the MSC response to statins was evaluated. The basic proliferation rate did not differ between cells from MSC-Y and MSC-A groups regardless of aerobic conditions. MSC-Y and MSC-A significantly increased the proliferation rate under

influence of bFGF both in atmospheric oxygen concentration and in hypoxia. In comparison to the untreated control bFGF increased proliferation in normoxia by 2.8-fold for MSC-Y and by 2.1 for MSC-A (for both groups  $p < 0.05$ ). There was no significant difference in response between groups. In hypoxia, MSCs from elder donors responded to bFGF significantly less than MSCs from younger donors (mean 2.6-fold increase for MSC-Y vs 1.7-fold increase for MSC-A,  $p = 0.04$ ) (Fig. 4). There was no significant difference between MSC-Y and MSC-A groups in response to atorvastatin and rosuvastatin regardless to aerobic conditions (Fig. 4). Raw data (mean OD450 and SEM) for each group are



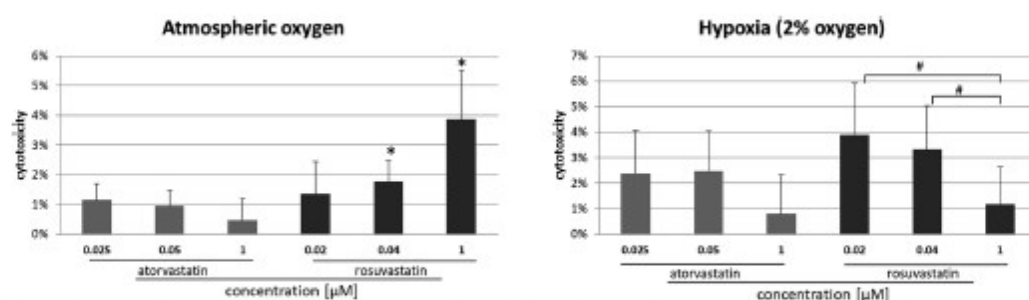


Fig. 3. The cytotoxic effect of statins on the of bone marrow mesenchymal stromal cells in different oxygen conditions (LDH test). The graphs present results in relation to the control untreated cells (CTRL) (expressed as percentages). The test assumes that CTRL value is 0% cytotoxicity. Data are presented as means and SEM. Statistical analysis was performed by Wilcoxon test or by T-test for relative values depending on data distribution.  $p < 0.05$  was assumed to be statistically significant. \* -  $p < 0.05$  in comparison to CTRL (low control in assay), # -  $p < 0.05$  – comparison between different treatments,  $N = 12$ .

presented in suppl. Table 2. Also, the results of LDH test did not differ between MSC-Y and MSC-A groups for any treatment.

#### 4. Discussion

In this first study evaluating the effect of rosuvastatin and atorvastatin on human bone marrow MSCs in different oxygenic conditions, we have observed that atorvastatin significantly increased proliferation rate of MSCs without significant cytotoxic effect irrespectively of oxygenic conditions. Rosuvastatin significantly increased MSCs proliferation rate without cytotoxic effect only in hypoxic conditions, whereas its application was associated with no change in proliferation rate but with significant cytotoxic effect in atmospheric oxygen concentration.

To date, the influence of statins on MSC observed in vitro has been described in several scientific papers. Among them, atorvastatin has been reported to reduce the apoptosis of oxygen-deprived animal bone marrow mesenchymal stem cells (BM-MSC) and specific mechanisms involved in this positive effect have been identified (Zhang et al., 2012; Dong et al., 2011; Li et al., 2014b). However, none of the researchers have evaluated the effect of atorvastatin combined with hypoxia on the proliferation of human MSC. Some researchers have explored the viability or proliferation rate of atorvastatin-treated human MSC at atmospheric oxygen concentration. For instance, Izadpanah et al. observed an increase in doubling time of human adipose tissue-derived MSC (AD-MSC) treated with atorvastatin at 65 ng/mL, which is almost twice higher than the dose evaluated in our proliferation assay (Izadpanah et al., 2015). Li et al. showed that atorvastatin may be associated with reduced human bone marrow MSC viability (Li et al., 2015). However, in this paper atorvastatin concentration (0.1–1.0 μM) which substantially exceeded maximal serum concentration used in our

experiment. Therefore, we hypothesize that the effect on MSC proliferation and viability exerted by atorvastatin is dose-dependent and only doses that do not exceed maximal serum concentration may be beneficial to human BM-MSC.

To the best of our knowledge, no study so far has evaluated the impact of rosuvastatin on human MSCs in hypoxia either. Based on observations in atmospheric oxygen concentration, Xu et al. found that pretreatment with rosuvastatin can significantly improve the viability of human MSC after exposure to TNF-α and the protective mechanism of rosuvastatin is partly mediated through miRNA-210 up-regulation (Xu et al., 2014). We have observed increased proliferation of rosuvastatin-treated MSC in hypoxia compared to atmospheric oxygen concentration. Moreover, in hypoxia the amount of released LDH was inversely proportional to the rosuvastatin concentration. The underlying mechanisms remain unknown, but this observation holds promise for MSC transplantation into ischemic tissues.

Myocardial ischemia appears especially suited for the statins combined with MSC application. The effect of atorvastatin and rosuvastatin with MSC transplantation had been evaluated on animal models of AMI in several studies with promising results (Yang et al., 2008; Tian et al., 2019; Zhang et al., 2014).

Guo et al. in the meta-analysis of preclinical studies on animal models, concludes that addition of atorvastatin to MSC therapy leads to significant improvement in left ventricular ejection fraction (Dai et al., 2015). Several biological pathways leading to these beneficial effects were identified including SDF-1/CXCR4 activation, RhoA/ROCK/ERK pathway inhibition, the increase of endothelial nitric oxide synthase expression in the peri-infarct zone and reduces MSC apoptosis (Yang et al., 2008; Tian et al., 2019; Zhang et al., 2014). The first human randomized, double-blind placebo-controlled study evaluating the

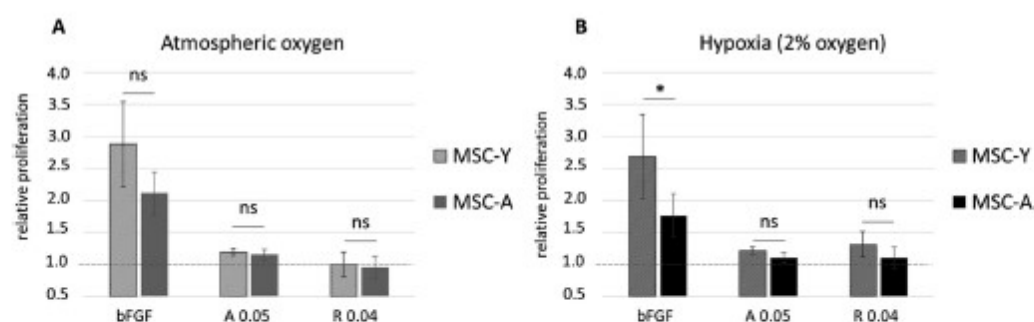


Fig. 4. The effect of bFGF and statins on the proliferation rate of bone marrow mesenchymal stromal cells from young and aged donors (BrdU test). The graphs present comparison of MSC proliferation depending on donor age. The relative proliferation is the ratio of cell proliferation after treatment to the untreated cells. Data are presented as means and SEM. Statistical analysis was performed by U Mann-Whitney test or T-test for unrelated data depending on data distribution.  $p < 0.05$  was assumed to be statistically significant. Y-MSC – young-MSC (cells from donors younger than 40 years old, mean 33,  $N = 6$ ), A-MSC – aged-MSC (cells from donors elder than 40 years, mean 67,  $N = 6$ ), bFGF – basic fibroblasts growth factor.



combination of oral atorvastatin with MSC injection in ST-elevation myocardial infarction is on-going (Xu et al., 2019).

The impact of MSC transplantation in combination with oral rosuvastatin supplementation on the infarcted murine hearts was also explored. Fu et al. reported that BMSC therapy with oral rosuvastatin administration promoted the survival of implanted cells by  $\approx 45$ -fold while compared with BMSC therapy alone. They observed that BMSC therapy with rosuvastatin (instead of BMSC therapy alone) upregulated the VEGF and bFGF expression, increased the capillary density and improved the cardiac function (Fu et al., 2016). Zhang et al. (2013) demonstrated that oral administration of rosuvastatin improved the survival of transplanted MSCs. Moreover, the authors have shown that in group receiving rosuvastatin, the proliferation of transplanted cells was significantly higher than in the group without rosuvastatin (based on co-localization of marker of grafted cells and Ki-67). These results are very consistent with our finding.

In our study we did not notice the significant difference in the basic proliferation rate between MSC-A and MSC-Y. Previously, many authors reported that proliferation activity of MSCs declines with age (Gnani et al., 2019; Mohd Ali et al., 2016). This inconsistency can be caused by two reasons. First, the difference of mean donors age between groups in our study was relatively small comparing to some other reports (Gnani et al., 2019). Second, we used the BrdU test which assesses proliferation over a short period of time. The results of data comparisons obtained i.e. during the evaluation of population doubling time may differ significantly from the BrdU test. Nevertheless, although we did not observed differences between MSC-Y and MSC-A in basic proliferation, we did noticed that cells from elder donor were less prone to respond to mitogenic factor (bFGF) than MSC-Y. In hypoxic conditions this difference was significant (Fig. 4). Age-dependent effect of statins on MSC proliferation was previously studied by Izadpanah et al. (Izadpanah et al., 2015). The authors did not report significant difference in the response to statins between MSCs from younger and elder donors. In our study we obtained similar results. Comparing the MSC responses to statins between the cells from younger and older donors no significant differences between the groups was noted (Fig. 4). However in hypoxia more distinct response to statins could be observed in cells from younger donors than from aged group. It seems that this issue requires further investigation on a larger sample size and more age-diverse donors.

Our study provides important data on human MSC, that merit further preclinical and clinical studies. Most of the studies evaluated oral statin administration, whereas it requires further verification whether MSC pretreatment or pretreatment combined with oral administration could yield better results. It could also be advantageous to apply hypoxia additionally to atorvastatin and, especially, rosuvastatin in the process of MSC preconditioning, as well as carefully choose the age of recipients. However, the mechanisms behind different response to rosuvastatin preconditioning in various oxygenic conditions and the impact of statins on secretory and migratory properties of MSC requires further investigation.

In conclusion, both atorvastatin and rosuvastatin at maximum serum concentrations increase MSC proliferation in hypoxic conditions without a significant change in cytotoxicity. Our results allow to propose a new explanation for the phenomenon previously observed in animal models in which statins enhanced the beneficial effects of MSC transplantation in cases of acute myocardial infarction.

#### Declarations of Competing Interest

None.

#### Funding

This study was financially supported by Medical University of Warsaw, Poland (Grant No. 1W21/NM1/17).

#### CRediT authorship contribution statement

**Damian Sienko:** Funding acquisition, Conceptualization, Investigation, Writing - original draft. **Dominka Klimczak-Tomanik:** Conceptualization, Investigation, Writing - review & editing. **Agnieszka Kulesza:** Investigation, Writing - review & editing. **Helena Symonides:** Investigation, Writing - original draft. **Marek Kuch:** Writing - review & editing. **Leszek Paczek:** Resources, Writing - review & editing. **Anna Burdzinska:** Investigation, Formal analysis, Visualization, Supervision, Writing - review & editing.

#### Acknowledgement

The authors would like to thank Paulina Gorka and Wiktorina Dawidczyk for help in identification of MSCs

#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.tice.2020.101427>.

#### References

- Back, K.H., Lee, W.Y., Oh, K.W., Tae, H.J., Lee, J.M., Lee, E.J., Han, J.H., Kang, M.I., Cha, B.Y., Lee, K.W., et al., 2005. The effect of simvastatin on the proliferation and differentiation of human bone marrow stromal cells. *J. Korean Med. Sci.* 20 (3), 438–444.
- Berglund, A.K., Schnabel, L.V., 2017. Allogeneic major histocompatibility complex-mismatched equine bone marrow-derived mesenchymal stem cells are targeted for death by cytotoxic anti-major histocompatibility complex antibodies. *Equine Vet. J.* 49 (4), 539–544.
- Berglund, A.K., Fortier, L.A., Antczak, D.F., Schnabel, L.V., 2017. Immunoprivileged no more: measuring the immunogenicity of allogeneic adult mesenchymal stem cells. *Stem Cell Res. Ther.* 8 (1), 288.
- Dai, G., Xu, Q., Luo, R., Gao, J., Chen, H., Deng, Y., Li, Y., Wang, Y., Yuan, W., Wu, X., 2015. Atorvastatin treatment improves effects of implanted mesenchymal stem cells: meta-analysis of animal models with acute myocardial infarction. *BMC Cardiovasc. Disord.* 15, 170.
- Dong, Q., Yang, Y., Song, L., Qian, H., Xu, Z., 2011. Atorvastatin prevents mesenchymal stem cells from hypoxia and serum-free injury through activating AMP-activated protein kinase. *Int. J. Cardiol.* 153 (3), 311–316.
- Fu, F.Y., Chen, B.Y., Chen, L.L., Zhang, P.L., Luo, Y.K., Jun, F., 2016. Improvement of the survival and therapeutic effects of implanted mesenchymal stem cells in a rat model of coronary microembolization by rosuvastatin treatment. *Eur. Rev. Med. Pharmacol. Sci.* 20 (11), 2368–2381.
- Gnani, D., Crippa, S., Della Volpe, L., Rossella, V., Conti, A., Lettera, E., Rivis, S., Omerti, M., Franchini, G., Bernardo, M.E., et al., 2019. An early-senescence state in aged mesenchymal stromal cells contributes to hematopoietic stem and progenitor cell clonogenic impairment through the activation of a pro-inflammatory program. *Aging Cell* 18 (3), e12933.
- Hu, X., Wu, R., Shehadeh, L.A., Zhou, Q., Jiang, C., Huang, X., Zhang, L., Gao, F., Liu, X., Yu, H., et al., 2014. Severe hypoxia exerts parallel and cell-specific regulation of gene expression and alternative splicing in human mesenchymal stem cells. *BMC Genomics* 15, 303.
- Hwang, N.S., Zhang, C., Hwang, Y.S., Varghese, S., 2009. Mesenchymal stem cell differentiation and roles in regenerative medicine. *Wiley Interdiscip. Rev. Syst. Biol. Med.* 1 (1), 97–106.
- Izadpanah, R., Schachtele, D.J., Pfnur, A.B., Lin, D., Slakey, D.P., Kadowitz, P.J., Alt, E. U., 2015. The impact of statins on biological characteristics of stem cells provides a novel explanation for their pleiotropic beneficial and adverse clinical effects. *Am. J. Physiol. Cell Physiol.* 309 (8), C522–531.
- Izad, M.M., Mazzarello, S., Zlepni, J., Dong, Y.Y.R., Montroy, J., McIntyre, L., Devereaux, P.J., Stewart, D.J., David Mazer, C., Barron, C.C., et al., 2018. Safety and efficacy of adult stem cell therapy for acute myocardial infarction and ischemic heart failure (SafeCell heart): a systematic review and meta-analysis. *Stem Cells Transl. Med.* 7 (12), 857–866.
- Li, Y., Charif, N., Mainard, D., Bensoussan, D., Stoltz, J.F., de Isla, N., 2014a. Donor's age dependent proliferation decrease of human bone marrow mesenchymal stem cells is linked to diminished clonogenicity. *Biomed. Mater. Eng.* 24 (1 Suppl), 47–52.
- Li, N., Zhang, Q., Qian, H., Jin, C., Yang, Y., Gao, R., 2014b. Atorvastatin induces autophagy of mesenchymal stem cells under hypoxia and serum deprivation conditions by activating the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway. *Chin. Med. J. (Engl.)* 127 (6), 1046–1051.
- Li, Y., Muller, A.L., Ngo, M.A., Sran, K., Bellan, D., Arora, R.C., Kirshenbaum, L.A., Freed, D.H., 2015. Statins impair survival of primary human mesenchymal progenitor cells via mevalonate depletion, NF-kappaB signaling, and Bnip3. *J. Cardiovasc. Transl. Res.* 8 (2), 96–105.

- Lin, C.S., Lin, G., Lue, T.P., 2012. Allogeneic and xenogeneic transplantation of adipose-derived stem cells in immunocompetent recipients without immunosuppressants. *Stem Cells Dev.* 21 (15), 2770–2778.
- Mohd Ali, N., Boo, L., Yeap, S.K., Ky, H., Satharasinghe, D.A., Liew, W.C., Ong, H.K., Cheong, S.K., Kamarul, T., 2016. Probable impact of age and hypoxia on proliferation and microRNA expression profile of bone marrow-derived human mesenchymal stem cells. *PeerJ* 4, e1536.
- Secunda, R., Vennila, R., Mohanashankar, A.M., Rajasundari, M., Jerwanth, S., 2015. Surendran R: isolation, expansion and characterisation of mesenchymal stem cells from human bone marrow, adipose tissue, umbilical cord blood and matrix: a comparative study. *Cytotechnology* 67 (5), 793–807.
- Tian, X.Q., Yang, Y.J., Li, Q., Xu, J., Huang, P.S., Xiong, Y.Y., Li, X.D., Jin, C., Qi, K., Jiang, L.P., et al., 2019. Combined therapy with atorvastatin and atorvastatin-pretreated mesenchymal stem cells enhances cardiac performance after acute myocardial infarction by activating SDF-1/CXCR4 axis. *Am. J. Transl. Res.* 11 (7), 4214–4231.
- van der Spoel, T.I., Jansen of Lorkeers, S.J., Agostoni, P., van Belle, E., Gyongyosi, M., Sluijter, J.P., Cramer, M.J., Doevendans, P.A., Chamuleau, S.A., 2011. Human relevance of pre-clinical studies in stem cell therapy: systematic review and meta-analysis of large animal models of ischaemic heart disease. *Cardiovasc. Res.* 91 (4), 649–658.
- Xu, J., Ren, D., Fu, M., Gao, Y., Lou, Y., Cai, S., Qian, J., Ge, J., 2014. [MicroRNA-210 mediates the protective effect of rosvastatin on human mesenchymal stem cells apoptosis induced by tumor necrosis factor- $\alpha$ ]. *Zhonghua xin xue guan bing za zhi* 42 (11), 932–937.
- Xu, J.Y., Qian, H.Y., Huang, P.S., Xu, J.Y., Xiong, Y.Y., Jiang, W.Y., Xu, J.Y., Leng, W.X., Li, X.D., Chen, G.H., et al., 2019. Transplantation efficacy of autologous bone marrow mesenchymal stem cells combined with atorvastatin for acute myocardial infarction (TEAM-AMI): rationale and design of a randomized, double-blind, placebo-controlled, multi-center, phase II TEAM-AMI trial. *Regen. Med.* 14 (12), 1077–1087.
- Yang, Y.J., Qian, H.Y., Huang, J., Geng, Y.J., Gao, R.L., Dou, K.F., Yang, G.S., Li, J.J., Shen, R., He, Z.X., et al., 2008. Atorvastatin treatment improves survival and effects of implanted mesenchymal stem cells in post-infarct swine hearts. *Eur. Heart J.* 29 (12), 1578–1590.
- Yang, Y.J., Qian, H.Y., Huang, J., Li, J.J., Gao, R.L., Dou, K.F., Yang, G.S., Willerson, J.T., Geng, Y.J., 2009. Combined therapy with simvastatin and bone marrow-derived mesenchymal stem cells increases benefits in infarcted swine hearts. *Arterioscler. Thromb. Vasc. Biol.* 29 (12), 2076–2082.
- Zanette, D.L., Lorenzi, J.C., Panepucci, R.A., Palma, P.V., Dos Santos, D.F., Prata, K.L., Silva Jr., W.A., 2015. Simvastatin modulates mesenchymal stromal cell proliferation and gene expression. *PLoS One* 10 (4), e0120137.
- Zhang, Q., Yang, Y.J., Wang, H., Dong, Q.T., Wang, T.J., Qian, H.Y., Xu, H., 2012. Autophagy activation: a novel mechanism of atorvastatin to protect mesenchymal stem cells from hypoxia and serum deprivation via AMP-activated protein kinase/mammalian target of rapamycin pathway. *Stem Cells Dev.* 21 (8), 1321–1332.
- Zhang, Z., Li, S., Cui, M., Gao, X., Sun, D., Qin, X., Narsinh, K., Li, C., Jia, H., Li, C., et al., 2013. Rosuvastatin enhances the therapeutic efficacy of adipose-derived mesenchymal stem cells for myocardial infarction via PI3K/Akt and MEK/ERK pathways. *Basic Res. Cardiol.* 108 (2), 333.
- Zhang, Q., Wang, H., Yang, Y.J., Dong, Q.T., Wang, T.J., Qian, H.Y., Li, N., Wang, X.M., Jin, C., 2014. Atorvastatin treatment improves the effects of mesenchymal stem cell transplantation on acute myocardial infarction: the role of the RhoA/ROCK/ERK pathway. *Int. J. Cardiol.* 176 (3), 670–679.



## 6.2. Allogenic Adipose-Derived stem cells in diabetic foot ulcer treatment: Clinical effectiveness, safety, survival in the Wound site, and proteomic impact



International Journal of  
Molecular Sciences



### Article

# Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact

Beata Mrozikiewicz-Rakowska <sup>1,†</sup>, Ilona Szablowska-Gadomska <sup>2,†</sup>, Dominik Cysewski <sup>3,4</sup>, Stefan Rudziński <sup>2</sup>, Rafał Płoski <sup>5</sup>, Piotr Gasperowicz <sup>5</sup>, Magdalena Konarzewska <sup>6</sup>, Jakub Zieliński <sup>7</sup>, Mateusz Mieczkowski <sup>8</sup>, Damian Sienko <sup>8,9</sup>, Tomasz Grzela <sup>10</sup>, Maria Noszczyk <sup>11</sup>, Barbara Paleska <sup>2</sup>, Leszek Czupryniak <sup>8</sup> and Małgorzata Lewandowska-Szumiel <sup>2,10,\*</sup>

<sup>1</sup> Department of Endocrinology, Centre of Postgraduate Medical Education, Bielanski Hospital, 01-809 Warsaw, Poland

<sup>2</sup> Laboratory for Cell Research and Application, Medical University of Warsaw, 02-097 Warsaw, Poland

<sup>3</sup> Clinical Research Centre, Medical University of Białystok, 15-276 Białystok, Poland

<sup>4</sup> Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-106 Warsaw, Poland

<sup>5</sup> Department of Medical Genetics, Warsaw Medical University, 02-106 Warsaw, Poland

<sup>6</sup> Department of Forensic Medicine, Warsaw Medical University, 02-007 Warsaw, Poland

<sup>7</sup> Interdisciplinary Centre for Mathematical and Computational Modelling, University of Warsaw, 02-106 Warsaw, Poland

<sup>8</sup> Department of Diabetology and Internal Diseases, Medical University of Warsaw, 02-097 Warsaw, Poland

<sup>9</sup> Doctoral School, Medical University of Warsaw, 02-091 Warsaw, Poland

<sup>10</sup> Department of Histology and Embryology, Medical University of Warsaw, 02-004 Warsaw, Poland

<sup>11</sup> Melitus Aesthetic Medicine and Anti-Aging Clinic, 01-627 Warsaw, Poland

\* Correspondence: mszumiel@wum.edu.pl

† These authors contributed equally to this work.



**Citation:** Mrozikiewicz-Rakowska, B.; Szablowska-Gadomska, I.; Cysewski, D.; Rudziński, S.; Płoski, R.; Gasperowicz, P.; Konarzewska, M.; Zieliński, J.; Mieczkowski, M.; Sienko, D.; et al. Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact. *Int. J. Mol. Sci.* **2023**, *24*, 1472. <https://doi.org/10.3390/ijms24021472>

Academic Editors: Francisco J. Vazoso and Noemi Elro

Received: 6 December 2022

Revised: 29 December 2022

Accepted: 10 January 2023

Published: 12 January 2023



**Copyright:** © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

**Abstract:** Although encouraging results of adipose-derived stem cell (ADSC) use in wound healing are available, the mechanism of action has been studied mainly in vitro and in animals. This work aimed to examine the safety and efficacy of allogenic ADSCs in human diabetic foot ulcer treatment, in combination with the analyses of the wound. Equal groups of 23 participants each received fibrin gel with ADSCs or fibrin gel alone. The clinical effects were assessed at four time points: days 7, 14, 21 and 49. Material collected during debridement from a subset of each group was analyzed for the presence of ADSC donor DNA and proteomic changes. The reduction in wound size was greater at all subsequent visits, significantly on day 21 and 49, and the time to 50% reduction in the wound size was significantly shorter in patients who received ADSCs. Complete healing was achieved at the end of the study in seven patients treated with ADSCs vs. one treated without ADSCs. One week after ADSC application, 34 proteins significantly differentiated the material from both groups, seven of which, i.e., GAPDH, CAT, ACTN1, KRT1, KRT9, SCL4A1, and TPI, positively correlated with the healing rate. We detected ADSC donor DNA up to 21 days after administration. We confirmed ADSC-related improvement in wound healing that correlated with the molecular background, which provides insights into the role of ADSCs in wound healing—a step toward the development of cell-based therapies.

**Keywords:** cell therapy; diabetic foot ulcer; adipose-derived stem cells; ADSC; ATMP; proteomics; clinical trial; allogenic cell therapy

## 1. Introduction

Diabetic foot ulcer (DFU) is one of the most common complications of poorly controlled diabetes mellitus [1]. Despite many efforts, the search for an effective therapy in the healing of DFU remains a very topical clinical issue and one of the major problems in the field of



chronic wounds [2,3]. Cell-based therapies using mesenchymal stem cells (MSCs) are of high interest due to their pro-regenerative and immunomodulatory properties [4]. MSCs were discovered in the bone marrow [5,6]; currently, they are obtained from various tissues, e.g., adipose tissue, umbilical cord, and even menstrual blood [7,8]. Adipose-derived stem cells (ADSCs) are one of the most common materials used for MSC therapy, as they are abundant, easy to obtain and have high immunomodulatory potency [4,9–12]. More than 300 clinical trials using ADSCs can be found at [clinicaltrials.gov](https://clinicaltrials.gov), while there is only one ADSC-based medicinal product registered in the EU as of now. There is some evidence that ADSCs can support wound healing through anti-inflammatory and proangiogenic effects and can enhance granulation tissue formation [9,13].

In 2019, Moon reported the successful treatment of DFU using allogenic ADSCs in a clinical trial. ADSCs were administered in the form of a hydrogel patch as a primary dressing, while in the control arm, only a secondary dressing was applied [14].

Encouraged by these results, we took the next step toward confirming and understanding the role of ADSCs as the active substance in the treatment of DFU. We also used allogenic ADSCs, though in our study design, the addition of these cells was the only variable examined. Furthermore, we tested for the presence of donor cells in the wound at subsequent time points and monitored molecular markers of ADSC-modulated wound healing by means of proteomic analysis of the recipient wound site. Currently, our knowledge about supportive MSC healing properties originates mainly from *in vitro* observations and animal studies. Therefore, we expect our approach to bring us closer to elucidating the mode of action of ADSCs in DFU treatment.

## 2. Results

### 2.1. Clinical Outcomes

Among 58 screened patients with neuropathic DFU (grades IA and IIA, according to the University of Texas classification), 47 individuals fulfilled the inclusion/exclusion criteria and were allocated to a study group (24 vs. 23 patients). In one patient initially allocated to the ADSC group, a small wound appeared on the contralateral leg 5 days after the application of ADSCs. This adverse reaction was assessed and determined as ADSC-independent. Following the exclusion criteria, this patient was excluded from the study group and was not included in the group statistics. Hence, both groups finally contained 46 patients, 23 each, who completed all planned medical procedures, including follow-up assessments. Notably, despite nonrandomized patient allocation, the study groups did not differ significantly in demographic or clinical baseline parameters (see Table 1). Moreover, they did not differ in wound area on the day of administration of fibrin gel with or without ADSCs (day 0) (average wound size 2.68 cm<sup>2</sup> vs. 2.72 cm<sup>2</sup>,  $p = 0.42$ ).

Table 1. Group characteristics.

|                  |           | ADSC<br>(N = 23) | Fibrin Gel<br>(N = 23) | Total<br>(N = 46) | <i>p</i> Value |
|------------------|-----------|------------------|------------------------|-------------------|----------------|
| Gender (N, %)    | M         | 20 (87%)         | 17 (74%)               | 37 (80%)          | 0.459          |
|                  | F         | 3 (13%)          | 6 (26%)                | 9 (20%)           |                |
| Age (years)      | Mean ± SD | 56.7 (11.1)      | 61.7 (7.5)             | 59.2 (9.7)        | 0.130          |
|                  | Median    | 56.0             | 63.0                   | 61.0              |                |
|                  | Min, Max  | 42.0, 75.0       | 38.0, 74.0             | 38.0, 75.0        |                |
| Body weight (kg) | Mean ± SD | 97.0 (20.4)      | 103.6 (18.2)           | 100.3 (19.4)      | 0.395          |
|                  | Median    | 98.0             | 105.0                  | 101.0             |                |
|                  | Min, Max  | 49.0, 130.0      | 68.0, 140.0            | 49.0, 140.0       |                |

Table 1. Cont.

|                                                |            | ADSC<br>(N = 23) | Fibrin Gel<br>(N = 23) | Total<br>(N = 46) | p Value |
|------------------------------------------------|------------|------------------|------------------------|-------------------|---------|
| Height (cm)                                    | Mean ± SD  | 179.7 (9.2)      | 177.1 (7.6)            | 178.4 (8.5)       | 0.499   |
|                                                | Median     | 178.0            | 179.0                  | 178.0             |         |
|                                                | Min, Max   | 160.0, 200.0     | 160.0, 188.0           | 160.0, 200.0      |         |
| Body mass index (kg/m <sup>2</sup> )           | Mean ± SD  | 30.0 (5.9)       | 33.0 (5.6)             | 31.5 (5.9)        | 0.068   |
|                                                | Median     | 30.8             | 33.0                   | 31.6              |         |
|                                                | Min, Max   | 19.1, 39.8       | 21.5, 49.6             | 19.1, 49.6        |         |
| Wound size in week 0 (cm <sup>2</sup> )        | Mean ± SD  | 2.72 (2.85)      | 2.68 (1.58)            | 2.70 (2.28)       | 0.420   |
|                                                | Median     | 1.9              | 2.20                   | 1.95              |         |
|                                                | Min, Max   | 1.0, 14.0        | 1.00, 5.6              | 1.00, 14.00       |         |
| Ulcer location (N, %)                          | Left limb  | 11 (48%)         | 11 (48%)               | 22 (48%)          | 1.000   |
|                                                | Right limb | 12 (52%)         | 12 (52%)               | 24 (52%)          |         |
|                                                | Forefoot   | 18 (78%)         | 16 (70%)               | 34 (74%)          | 0.099   |
|                                                | Hindfoot   | 0 (0%)           | 4 (17%)                | 4 (9%)            |         |
|                                                | Midfoot    | 5 (22%)          | 3 (13%)                | 8 (17%)           |         |
|                                                | Plantar    | 17 (74%)         | 20 (87%)               | 37 (80%)          | 0.486   |
|                                                | Dorsal     | 3 (13%)          | 2 (9%)                 | 5 (11%)           |         |
|                                                | Both       | 3 (13%)          | 1 (4%)                 | 4 (9%)            |         |
| Duration of diabetic foot ulcers (months)      | Mean ± SD  | 19.5 (31.1)      | 30.2 (35.9)            | 24.9 (33.6)       | 0.155   |
|                                                | Median     | 9.0              | 14.0                   | 12.0              |         |
|                                                | Min, Max   | 1.0, 144.0       | 1.0, 156.0             | 1.0, 156.0        |         |
| Duration of diabetes mellitus (years)          | Mean ± SD  | 16.1 (8.6)       | 19.7 (10.7)            | 17.9 (9.8)        | 0.424   |
|                                                | Median     | 16.0             | 18.0                   | 16.0              |         |
|                                                | Min, Max   | 2.0, 39.0        | 3.0, 44.0              | 2.0, 44.0         |         |
| DM type (n, %)                                 | Type 1     | 4 (17%)          | 5 (22%)                | 9 (20%)           | 0.361   |
|                                                | Type 2     | 15 (65%)         | 17 (74%)               | 32 (70%)          |         |
|                                                | Other      | 4 (17%)          | 1 (4%)                 | 5 (11%)           |         |
| Neuropathy (N, %)                              | Yes        | 23 (100%)        | 23 (100%)              | 46 (100%)         | —       |
|                                                | No         | 0 (0%)           | 0 (0%)                 | 0 (0%)            |         |
| Retinopathy (N, %)                             | Yes        | 14 (61%)         | 13 (57%)               | 27 (59%)          | 0.811   |
|                                                | No         | 9 (39%)          | 10 (43%)               | 19 (41%)          |         |
| Coronary artery disease (N, %)                 | Yes        | 3 (13%)          | 6 (26%)                | 9 (20%)           | 0.459   |
|                                                | No         | 20 (87%)         | 17 (74%)               | 37 (80%)          |         |
| Hypertension (N, %)                            | Yes        | 16 (70%)         | 22 (96%)               | 38 (83%)          | 0.130   |
|                                                | No         | 7 (30%)          | 1 (4%)                 | 8 (17%)           |         |
| Smoking history (N, %)                         | Yes        | 15 (65%)         | 14 (61%)               | 29 (63%)          | 0.811   |
|                                                | No         | 8 (35%)          | 9 (39%)                | 17 (37%)          |         |
| ABI                                            | Mean ± SD  | 1.05 (0.14)      | 1.11 (0.18)            | 1.08 (0.16)       | 0.130   |
|                                                | Median     | 1.00             | 1.08                   | 1.00              |         |
|                                                | Min, Max   | 0.83, 1.40       | 0.70, 1.45             | 0.70, 1.45        |         |
| tPO2                                           | Mean ± SD  | 54.6 (19.3)      | 52.3 (17.9)            | 53.4 (18.4)       | 0.695   |
|                                                | Median     | 53.0             | 50.0                   | 50.5              |         |
|                                                | Min, Max   | 25.0, 98.0       | 25.0, 87.0             | 25.0, 98.0        |         |
| Glycated hemoglobin A1c at screening visit (%) | Mean ± SD  | 7.25 (1.31)      | 7.57 (1.35)            | 7.41 (1.33)       | 0.536   |
|                                                | Median     | 7.40             | 7.30                   | 7.40              |         |
|                                                | Min, Max   | 5.00, 10.70      | 5.40, 10.80            | 5.00, 10.80       |         |

Table 1. Cont.

|                                   |               | ADSC<br>(N = 23) | Fibrin Gel<br>(N = 23) | Total<br>(N = 46) | p Value |
|-----------------------------------|---------------|------------------|------------------------|-------------------|---------|
| Creatinine level (mg/dL)          | Mean $\pm$ SD | 1.11 (0.28)      | 1.17 (0.38)            | 1.14 (0.33)       | 0.828   |
|                                   | Median        | 1.11             | 1.05                   | 1.07              |         |
|                                   | Min, Max      | 0.64, 1.60       | 0.74, 1.87             | 0.64, 1.87        |         |
| GFR (mL/min/1.73 m <sup>2</sup> ) | Mean $\pm$ SD | 74.8 (21.2)      | 71.3 (24.4)            | 73.0 (22.7)       | 0.811   |
|                                   | Median        | 78.0             | 74.0                   | 76.0              |         |
|                                   | Min, Max      | 45.0, 113.0      | 27.0, 105.0            | 27.0, 113.0       |         |

Data for patients' clinical parameters mean (SD), median, minimum and maximum. In the last column are the results of the Wilcoxon test (for quantitative variables) and Fisher's exact test (for categorical variables) comparing the ADSC and the fibrin gel groups. None of the differences fulfilled the  $p < 0.05$  cutoff point. DM—diabetes mellitus; ABI—ankle brachial index; GFR—glomerular filtration rate; tPO2—transcutaneous oxygen tension.

The time to 50% reduction in the size of the wound in the group receiving fibrin gel was  $25.5 \pm 4.2$  days and in the ADSC-group it was  $17.6 \pm 1.5$  days ( $p = 0.029$ ). Figure 1a compares the time of wound area reduction based on the changes in wound size measured on a weekly basis. Additionally, photographic documentation was taken to illustrate the changes in patients wounds. Figure 1b shows exemplary photographs of wounds in the studied groups (for photographic documentation from the complete set of patients see Figure S1). The wound size quotient at subsequent visits was smaller in the ADSC group than in the gel group. The differences were not significant on day 7 and 14 ( $p = 0.109$  and  $0.055$ , respectively), while they were significant ( $p = 0.019$  and  $p = 0.023$ ) on day 21 and 49. The results are presented in Figure 1a. At the end of the study the wound completely healed in seven patients from the ADSC group and in only one patient from the gel group. The evaluation of pain sensation did not differ significantly between the groups (Table S1). The lack of differences in pain between the study groups indicates that patients were appropriately selected for the study groups due to the inclusion criteria—all were characterized by an established form of neuropathy. The occurrence of pain in such a patient could be indicative of a significantly increased inflammatory process, which was an exclusion criterion for the study. As for the possible effect of ADSCs on nerve regeneration, at this stage of neuropathy, the reversal of damage to the vasa nervorum, and therefore nerve fiber reinnervation, has not yet been described in humans, and further research is needed. For example, analysis of the SDF-1/CXCR4, a pathway important for the process of neovascularisation, which is of critical importance for neuroregeneration in DFU, may be taken into account, as well as its changes under the influence of stem cells and factors secreted by them [15,16].

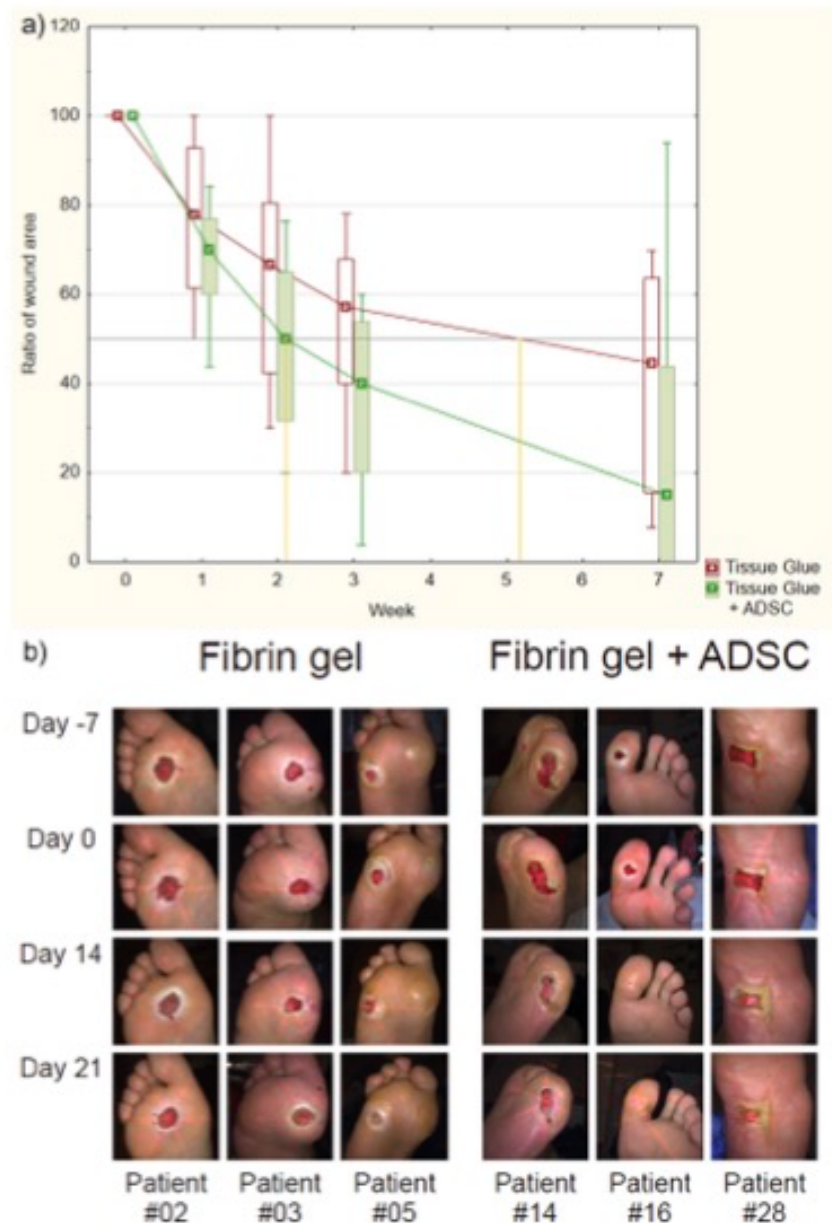
The groups had similar rates of adverse events (Table S2). None of them were related to the medical procedures that the patients underwent. No serious adverse events were observed.

In general, the ADSC group benefited from the use of stem cells in terms of accelerated wound healing and had a greater likelihood of complete wound healing.

## 2.2. Proteomic Analysis

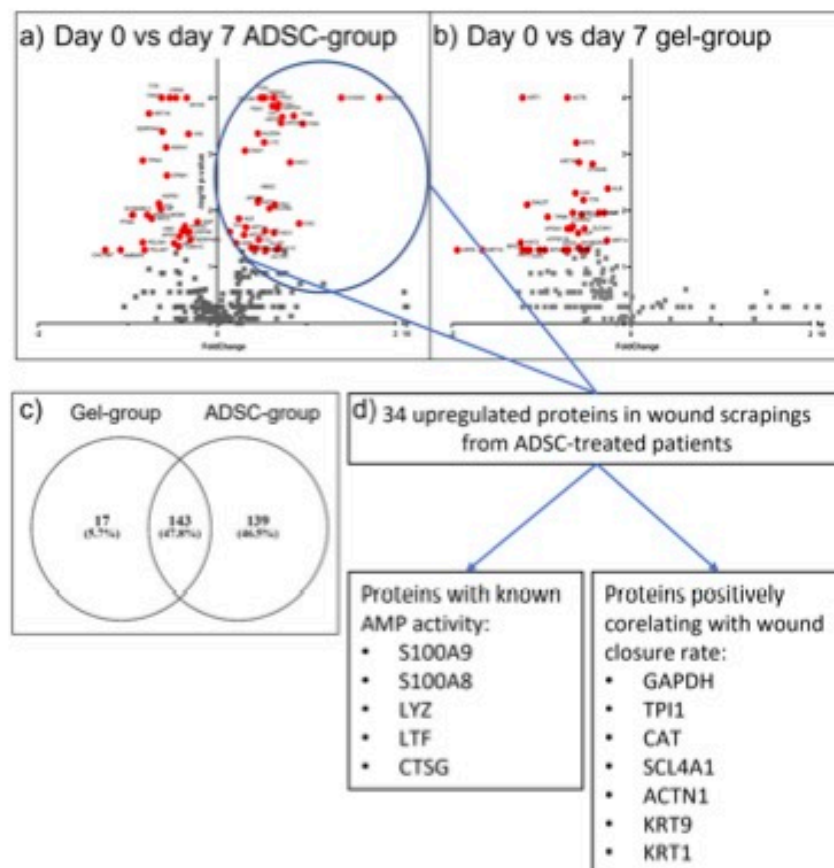
We identified and quantified over 300 proteins in wound scrapings from each of the three LC-MS/MS groups. The samples were characterized by high heterogeneity and a wide range of protein concentrations. Sets 2 and 3 were merged for analysis. In the ADSC group, 34 proteins statistically significantly differentiated the day 0 and day 7 time points ( $p < 0.05$ , Benjamin-Hochberg correction) (Figure 2a–c). The identified enriched proteins are functionally connected (Figure S4). The Reactome platform identified several processes, such as antimicrobial peptide (AMP) synthesis (Table S3), integrin signaling, and amyloid formation (Figure S2) [17]. We further compared the upregulated proteins with the course of the treatment in the ADSC group patients and found that seven of the identified

proteins—GAPDH, TPI1, CAT, SCL4A1, ACTN1, KRT9 and KRT1 positively correlated with the wound closure rate (Table S4).



**Figure 1.** Comparison of fibrin gel group and ADSC group patients' course of treatment: (a) Time dependence of the relative size of the wound in both groups. Relative wound size is defined as the ratio of the wound size at a given week to the size at week zero. At week zero, patients received either tissue glue or tissue glue along with ADSC; (b) Photographs of wound areas in the studied groups illustrating the course of DFU treatment in the study and the difference between patients from the fibrin gel group and the ADSC group. Photographs were taken in weekly intervals starting from the screening visit (Day-7), treatment application (Day 0), and two and three weeks into the treatment (Day 14 and Day 21, respectively).





**Figure 2.** Results of wound scrapings proteomic analysis—Volcano plot of the identified protein intensities: (a) ADSC group day 0 vs. day 7; (b) Fibrin gel group day 0 vs. day 7; (c) Venn diagram comparing identified proteins in the ADSC group and fibrin gel group in both day 0 and day 7; (d) Position at volcano-plot and highlighted antimicrobial proteins and proteins correlating positively with wound healing.

Summing up, we not only discovered a panel of proteins which differentiated wound scrapings from the ADSC group and fibrin gel group, but we also found out that seven of them were positively correlated with the progress of healing.

### 2.3. Donor DNA in Wound Samples

Wound scraping samples for donor DNA admixture assessment were collected from 21 patients from the ADSC group at three time points—day 7, day 14 and day 21. Of all 63 recipient wound scraping samples (three time points per patient) from the ADSC group, donor DNA was detected in eight and nine samples using STR and ADS, respectively. Both methods detected the admixture in the same 8 scraping samples, while one more sample was positive by ADS. Donor DNA was mainly found in samples from day 7 (in 7 out of 21 patients), but single samples from day 14 and day 21 time points were also positive. The amount of donor DNA ranged from 1.4 to 4.7% of the total DNA. In the two scraping samples, where donor DNA was detected on day 14 (patient 1ABC01019) or on day 21 (patient 1ABC01027), the amount present was similar to (for 1ABC01019) or higher than (for 1ABC01027) that in the same patient on day 7 (Table S5). However, no donor DNA was detected in samples from patient 1ABC01027 collected on day 14. An analysis of the amount of donor DNA detected through STR loci with relatively long and short



amplicon sizes did not show consistent differences (Figure S3). Only in one sample (sample 1ABC01019, day 14) did we find a significant difference ( $p = 0.0041$ ), indicating a higher result with the short loci (4.6%) than the long loci (1.9%). In other samples the trends were mixed. In four samples there was a trend for higher values at the short loci; in two samples the opposite was true; and in one sample the values were virtually identical. When samples were pooled, there was no significant difference between the mean values for the short and long loci (0.042 vs. 0.038, respectively,  $p = 0.28$ , for raw data see Tables S5–S8).

Taken together, by using two independent methods, we were able to confirm the presence of donor DNA in the samples of the treated wounds. Therefore, we have shown that ADSCs are capable of migrating from the fibrin gel into the wound bed. Moreover, the STR analysis results are compatible (although they clearly do not constitute proof) with the presence of at least some living donor cells at the time of sampling.

### 3. Discussion

DFU poses a serious clinical threat, so there is a need for new therapeutic approaches, such as advanced wound healing technologies that harness the pro-regenerative and immunomodulatory potential of ADSCs. We were inspired by Moon et al., who provided evidence of allogenic ADSCs' safe and effective use in the treatment of DFU when applied to the wound in a hydrogel patch as a primary dressing [14]. In our study, fibrin gel was administered with or without ADSCs in suspension, allowing us to confirm Moon's findings under an experimental setup in which the presence of cells, as an active substance, was the only variable. This design was necessary to distinguish their role in wound healing. Our observations also differ from Moon's study in that Moon et al. used cells frozen in a gel dressing, whereas in our work, cells were frozen, thawed and cultured before application in the fibrin gel. Therefore, we were able to examine cells' viability before application, thus providing better control over the medicinal product's quality as well as the similarity of subsequent product batches. Moreover, Moon et al.'s control group was treated solely with a polyurethane foam dressing, whereas in our work the same fibrin gel was applied in both groups. Taking into account the composition of the hydrogel used by Moon et al. was not provided, the influence of the hydrogel itself on the healing process cannot be excluded. Thus, our observation is a step forward. It supports Moon's observation of the safety of using allogenic ADSCs in DFU—no adverse effects were connected with the procedure. We also demonstrated ADSC efficacy in terms of the time needed to reduce the wound area by 50% from the initial size, the wound size decrease at each visit, and the number of patients who achieved fully closed wounds within the time frame of the study. These findings were obtained from a nonrandomized and nonblinded study, which is a limitation of our work, even though the groups being compared did not differ significantly in demographic or clinical parameters at baseline.

On the other hand, while Moon focused on the clinical effect, we also tried to get closer to assessing the impact of the cells themselves at the molecular level. Our donor ADSC detection in the wound site as well as our proteomic analysis of wound scraping samples are novelties of this work. The presence of the ADSC donor's genetic traces was assessed by two independent methods: STR and ES/ADS. Both methods showed that the DNA traces of donor cells were found even 21 days after administration of the medicinal product—in this case, in one sample, patient 1ABC01027 (see Table S5). To date, cell viability after ADSC administration has been observed only in animal models [18,19], but our results show that ADSCs migrated from the fibrin gel into the wound site. Moreover, the low degradation of STR markers suggests that the DNA traces found in the wound scraping samples up to 21 days after application could come from viable ADSCs. Our observations confirm the worth of ADSC therapy and suggest that it may exert a direct impact on the course of wound healing in DFU even 21 days after application. Although we found ADSC donor genetic traces in only about 30% of patients at different time points after application, mainly on day 7, one should keep in mind that they were observed in wound scraping samples obtained during routine wound cleanup. While these procedures are meant to only remove

fragments of unhealed tissue, we assume that ADSCs could have accumulated in healthy tissue that had already undergone epithelialization.

In fact, if we didn't find traces of the donor material in the scrapings at all, it still would not rule out the presence of donor material in the healed part of the wound. Interestingly, in patient 1ABC01027, where donor DNA was detected on day 21, there were no donor DNA traces found in the sample from day 14. It seems possible that ADSCs were present in the healing part of the wound on day 14 and then migrated from there to become detectable in scrapings on day 21. Taking into account the low degradation of STR markers, we may assume that the detected cells were alive and, as such, capable of proliferation and migration. This would explain the similar or even higher amounts of DNA detected in some cases at later time points compared to earlier ones (patients 1ABC01019 or 1ABC01027). Nevertheless, such interpretations are only speculative. What we really proved was the presence of ADSCs donor DNA, at various time points from application, in human clinical observation, which is an important contribution to our knowledge of cell-based therapies.

We identified 34 proteins that were highly overrepresented or identified exclusively in samples from day 7 after ADSC application, compared to day 0 (before application). We observed elevated levels of APOA1 and PFN1 proteins in the ADSC group, proteins reported to play a role in proangiogenic processes, either by facilitating vascular endothelial cell migration (PFN1) [20] or the promotion of proliferation and differentiation of human endothelial progenitor cells (APOA1) [21]. Interestingly, increases in the levels of GAPDH, CAT, ACTN1, KRT1, KRT9, SCL4A1, and TPI correlated with the rate of wound healing by the individual patient (Figure 2d and Table S4). The immunomodulatory characteristics of GAPDH [22] and CAT proteins have been shown in humans and in animal models, respectively [23]. These proteins are involved in macrophage polarization from the M1 to the M2 phenotype and in the inflammation-to-proliferation phase shift in wound healing, a process crucial to wound healing progress.

The overrepresentation of KRT1 and KRT9, which are specific to keratinocytes, indicates ongoing regenerative processes, as keratinocytes are not present in the native chronic wound bed [24]. ACTN1 regulates the directional migration of keratinocytes [25]. The increase in these molecules might be considered a molecular marker of re-epithelialization. It is noteworthy that the increase in the levels of these three proteins correlated positively with the wound closure rate in the ADSC group. This finding might suggest that KRT1, KRT9, and ACTN1 could become prognostic markers for ADSC-based therapies in DFU treatment. This possibility should be tested in future validation studies.

To better understand the ADSCs' influence on wound healing, we performed a pathway analysis with the Reactome platform. We focused on the highest-probability identified pathways ( $p < 0.0001$ , FDR 0.01%). A number of processes might be connected to the healing process (e.g., amyloid fiber formation, plasma lipoprotein remodeling, integrin signaling). Others, such as antimicrobial peptides, play a supportive role in the process, e.g., modulating chronic inflammation, mainly by wound microbiome control [26]. Sequential activation of the processes that must occur in the wound healing process is, in our opinion, additional proof that the obtained data are not random findings. Interestingly, 40% of proteins observed in increased levels have or are predicted to have AMP properties. Five proteins were already annotated in databases as AMPs (Figure 2d), and nine others were highly scored by AmpGram to have AMP probability [27] (Table S3). Keeping potentially harmful microbiota at bay may be one of the factors that enhance wound healing [28,29]. These findings show the variety of wound healing-accelerating effects generated by ADSCs.

## 4. Materials and Methods

### 4.1. Clinical Observation Design

The study was performed under hospital exemption in patients with DFU of neuropathic origin for whom standard wound healing treatment had failed. It was a prospective parallel-group trial without randomization and without blinding. Patient preselection was based on the inclusion/exclusion criteria described below. This clinical observation

was performed with a strict adherence to “STROBE” (Strengthening The Reporting and OBservational studies in Epidemiology) guidelines.

The patient inclusion criteria were as follows: age >18; chronic wound area of 1–25 cm<sup>2</sup>; glycated hemoglobin (HbA1c) <11%; transcutaneous oxygen tension (tPO2) ≥30 mmHg in the wound area or systolic pressure on the distal tibial arteries ≥50 mmHg; and general condition of the patient that in the opinion of the investigator allowed participation in all study procedures.

The patient exclusion criteria were ulcer etiology other than DFU; wound area >25 cm<sup>2</sup>; HbA1c ≥11%; clinically significant limb ischemia as shown by tPO2 <30 mmHg or arterial pressure on the distal tibial arteries <50 mmHg; active wound infection; allergy to thrombin; active venous thrombosis; presence of the wound on the contralateral foot; any systemic disease in the stage of exacerbation (acute or uncontrolled); and any oncological treatment within the last 5 years.

The early study termination conditions were as follows: the withdrawal of the patient's consent to participate in the study, significant violations of the study protocol by the patient or investigator, and any condition where (upon individual decision of the investigator) further participation in the study could pose any risk to the patient.

Patients who fulfilled all inclusion criteria and did not meet any exclusion criteria were allocated to one of two study groups as follows. The first, called the fibrin gel group, was treated with fibrin gel covered with a secondary dressing, and the second, the ADSC group, was treated with ADSCs suspended in fibrin gel and secured with a secondary dressing. Although the study was not randomized, we confirmed that the two groups were similar at baseline. After the study, demographic and clinical parameters were compared to detect any differences that might have arisen between them.

The volunteers gave their informed consent to participate in the trial. The Bioethics Committee at the Medical University of Warsaw approved the trial (approval no. KB/128/2019 and KB/3/A2021), and the trial was conducted according to the ethical guidelines of the World Medical Association Declaration of Helsinki. The clinical part of the study was registered at ClinicalTrials.gov (identifier: NCT03865394) and ran from October 2019 to July 2021 in the Department of Diabetology and Internal Diseases of the Medical University of Warsaw.

The clinical outcomes were the wound healing rate and the safety of the applied therapy. The former was assessed as the time needed to reduce the wound area by 50% from the initial size, determined by calculating the relative size of the wound at each visit to the size on the day of application of fibrin gel with or without ADSCs, and the number of patients who achieved a fully closed wound within the time frame of the study. Molecular assessment of the therapeutic efficiency was accomplished by analyzing the expression of selected proangiogenic and immunomodulatory factors and detecting donor DNA traces in the wounds of both study groups.

#### 4.2. ADSC Collection and Preparation

ADSCs from one healthy donor were used in order to avoid any donor-related variability in results. The informed consent of the donor, approved by the Bioethics Committee (opinion nos. KB/128/2019 and KB/3/A2021), was obtained. Medical qualification of the donor was based on the rules determined by a transplantation law. ADSCs for clinical application were produced in the Laboratory for Cell Research and Application, Medical University of Warsaw, in compliance with the GMP standard laid down for Advance Therapy Medicinal Products (ATMP) with the consent of the Chief Pharmaceutical Inspectorate (consent no. 01/0556/2019). Lipoaspirate obtained from the abdominal adipose tissue at the Mellitus Clinic was used as a starting material. After isolation, cells were cultured to obtain MSCs and stored frozen in liquid nitrogen. Prior to application, the cells were thawed, briefly cultured and then used up to the third passage.

For the ADSCs isolation, collagenase NB 6 GMP Grade (Nordmark Pharma GmbH, Uetersen, Germany) solution was used in the proportion of adipose tissue to collagenase equal to 1:1 (v/v). The collagenase treatment was carried out for 1 h at 37 °C under constant



shaking. Prior to digestion, the adipose tissue was washed extensively with 1% antibiotic-antimycotic solution (Corning Inc., Corning, NY, USA) in phosphate-buffered saline (PBS) (ThermoFisher Scientific, Waltham, MA, USA). To get rid of the blood cells, ZAPR™ (Incell, Frisco, TX, USA) a buffer for lysis of erythrocytes, was used according to the manufacturer's recommendations. The cells were then separated by centrifugation (at  $300\times g$  for 5 min at RT), rinsed with fresh NutriStem® XF Basal Medium, and filtered through a cell strainer. After isolation cells were counted with an ADAM-MC automated cell counter (NanoEnTek Inc., Seoul, Republic of Korea), plated in T75 culture flasks and cultured at 37 °C and 5% CO<sub>2</sub> in a humidified atmosphere in the complete culture medium MSC NutriStem® XF Basal Medium with Supplement Mix (Biological Industries, Beit-Haemek, Israel) and 0.1% antibiotic-antimycotic solution (Corning Inc., Corning, NY, USA). MSC NutriStem® is a xeno-free and serum-free medium devoted to translational use for cell cultures carried out under the Good Manufacturing Practice (GMP) regime. Isolated cells were expanded in culture using the same conditions. Cells from the first and the second passage were stored frozen in liquid nitrogen.

In order to confirm the desired immunophenotype of the cells, a common set of positive and negative cell surface markers were determined by means of cytometric analysis [30]. Conjugated mouse monoclonal antibodies directed against CD29, CD31, CD34, CD45, CD73, CD105 and CD146 (ThermoFisher Scientific, Waltham, MA, USA) were used, and the analysis was performed by using a CytoFlex Beckman Coulter flow cytometer. More than 98% of the population was CD105+, CD73+ and CD29+, CD31−, CD45−, and CD146−, and more than 90% of the cells were CD34−. Overall, FACS analysis revealed that 98.66% of the total population of analyzed cells fulfilled the minimal criteria for ADSCs.

#### 4.3. Manufacturing of the Medicinal Product

Prior to application, the cells were thawed, cultured in complete MSC NutriStem® XF Basal Medium with Supplement Mix (Biological Industries, Beit-Haemek, Israel) and 0.1% antibiotic—antimycotic solution (Corning Inc., Corning, NY, USA) until appropriate confluence (2–3 days cultured) and passaged—only cells from the second or third passage were used for the final product. The cells were then detached using StemPro™ Accutase™ Cell Dissociation Reagent (ThermoFisher Scientific, Waltham, MA, USA). The cells were then rinsed twice with sodium chlorate (Fresenius Kabi, Warsaw, Poland), and they were centrifuged at  $350\times g$  for 5 min at 22 °C after each rinse. Cell number and viability were then assessed (automated cell counter ADAM-MC, NanoEnTek Inc., Seoul, Republic of Korea). Finally, each individual batch released to the clinic consisted of a 1 ml suspension of  $2.5 \times 10^6$  ADSCs in sodium chloride with a cell viability greater than 70%.

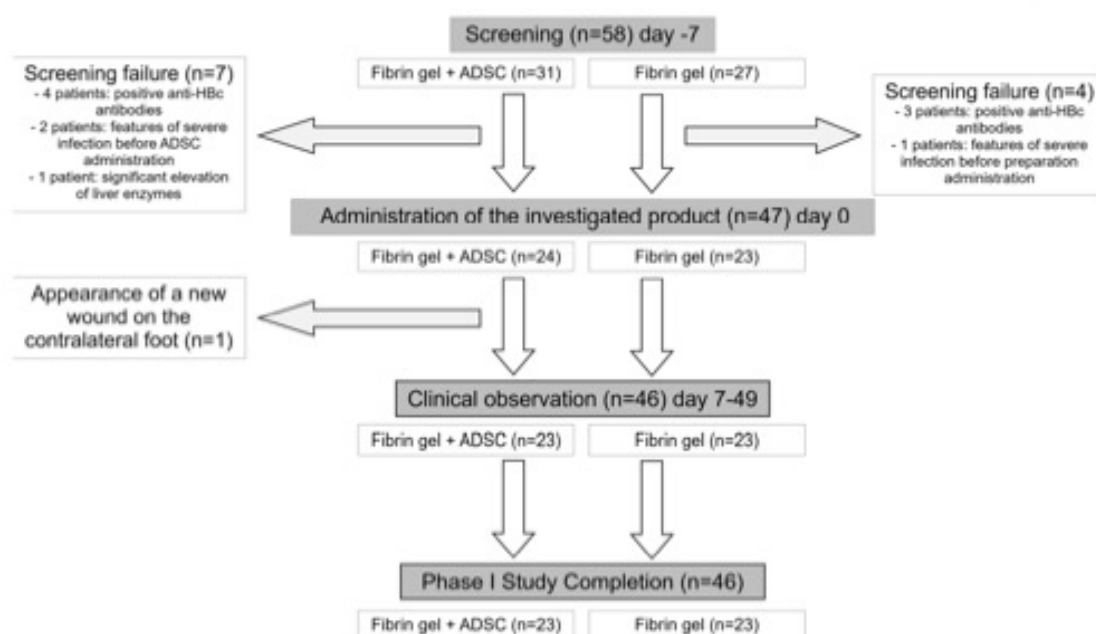
Additionally, to assure patient safety, samples were taken several times during the manufacturing process for analysis to confirm sterility and to test the level of endotoxins according to the GMP rules specific to ATMPs.

#### 4.4. Clinical Procedures

At the screening visit (day-7), all eligible patients were subjected to verification of the inclusion/exclusion criteria. The main criteria for participation in the study included wound size, assessed using a Silhouette® laser scanner; tPO2, measured by a TCM400 device; systolic pressure in the distal limb arteries; and other clinical features described in the study protocol. Furthermore, blood samples were taken for basic laboratory and viral tests. The same procedures were repeated during a visit on day 0 before the application of the tested formulation to the wound.

In the ADSC group, the cell suspension in fibrin gel was applied directly onto the wound using a special applicator. In order to ensure the required quality of the fibrin gel, the commercially available medicinal product TISSEEL Lyo (Baxter, Deerfield, IL, USA) was used. According to the manufacturer's instructions, the two components of the product, i.e., the Sealer Protein (Human Fibrinogen and Aprotinin) Solution and Thrombin Solution can be applied using a Duploject system, which allows for the combination of the two

components during application. For the ADSC group, cell suspension in NaCl 0.9%, as described above, was added to each component of the system in equal proportions, mixed, and applied to the wound using a Duploject system. For the patients who received fibrin gel only (Fibrin gel group), the same procedure was used with the addition of NaCl 0.9%, without cells as the only difference. For wounds up to 12 cm<sup>2</sup>,  $2.5 \times 10^6$  cells in 1 ml of fibrin gel were used, whereas for wounds above 12 cm<sup>2</sup>, the volume of cell suspension was increased proportionally. Patients were administered this formulation only once, during a visit on day 0. The wound was then covered with a secondary dressing (UrgoTul, Laboratoires Urgo, Chenôve, France). That dressing was changed every  $3 \pm 1$  days, and control hospital visits were every  $7 \pm 3$  days. The treatment efficacy was assessed at weekly intervals (days 7, 14, 21) and on day 49. The same procedures, except for the ADSC admixture to the fibrin gel suspension, were applied in the gel group (Figure 3).



**Figure 3.** A consort diagram illustrating the flow of patients included in the study. Gray boxes represent the main stages of the study, with the total number of patients at the respective study point; vertical arrows illustrate the flow of patients between them. White frames below show the number of individuals in the respective treatment arms (“Fibrin gel + ADSC” or “Fibrin gel” alone), at each study stage. The drop-out cases are shown in boxes lateral to the horizontal arrows.

Patient participation in the study was terminated when the wound was healed or 49 days after the application, whichever came first. If the wound was not completely healed 49 days after application, the patient was further treated in compliance with the general principles of chronic wound care.

#### 4.5. Evaluation of the Clinical Outcomes

The clinical effectiveness and safety of treatment were assessed at each visit through the following:

- measurement of the wound surface;
- local and systemic tolerance of therapy, including the episodes of wound infection or any other adverse events;
- number of patients in each group who achieved complete wound closure;



- evaluation of the pain scale: evaluation of the changes in pain experienced by the patient due to the wound treated with the tested method, in relation to the wound treated in a standard way through the use of the visual analog scale (VAS).

#### 4.6. Wound Debridement

According to the TIME recommendations, to remove any unvital or contaminated material, the wound was cleaned up using a Volkmann spoon curette during each visit until the wound was completely healed. Part of this waste material (hereafter, scrapings), particularly those removed from the bottom of the ulcer, was deep-frozen and stored at  $-80^{\circ}\text{C}$  until further molecular assessments were undertaken. The above debridement was not performed after the wound had completely healed.

#### 4.7. Proteomic Analysis

Each wound scraping sample was dissolved in a lysis buffer, denatured and sonicated. Debris was removed by centrifugation. Proteins were then precipitated with a modified methanol/chloroform protocol [31].

The pellet of extracted proteins was digested with LysC/trypsin as described [32] and labeled with iTRAQ8-plex. Samples were organized into three sets for LC-MS/MS analysis: set 1: scrapings samples of three patients from the fibrin gel group at two time points (day 0, day 7); sets 2 and 3: scrapings samples of six patients from the ADSC group at two time points (day 0, day 7) analyzed as two separate groups of three. Combined samples were prefractionated and analyzed by LC-MS/MS online UPLC and a QExactive Orbitrap mass spectrometer at the Institute of Biochemistry and Biophysics PAS. Data were searched using the MaxQuant platform against UniProt reference proteome database taxonomy for statistical analysis with Scaffold Q+S. For the detailed protocol, see Description S1. Data were deposited in the PRIDE repository under access number PXD032099.

#### 4.8. Search for Donor DNA in Wound Scrapings

Seventy wound scraping samples were analyzed (63 samples collected from 21 recipients in the ADSC group at three time points (day 7, day 14, day 21)), six negative controls collected from two recipients in the gel group and one positive control (donor). We searched for donor DNA in wound samples by two methods: (i) short tandem repeat (STR) analysis; and (ii) amplicon deep sequencing (ADS) designed to contain a rare variant (A/G at rs35874463) selected after exome sequencing (ES) of the donor. The size of donor DNA admixture was estimated as a percentage of total DNA in the scraping samples by measuring the areas under the peak (STR analysis) or as the proportion of variant reads (amplicon sequencing). Donor DNA degradation was assessed by analyzing whether the amount of donor DNA detected was higher when shorter vs. longer STR alleles were considered. Detailed methods are described in Description S2.

#### 4.9. Statistical Analyses

To compare the ADSC and fibrin gel groups before treatment, the Wilcoxon test (for quantitative variables) and Fisher's exact test (for categorical variables) were used. The group comparison results are summarized in Table 1.

Significantly changed proteins in the proteomics experiment were those with  $p < 0.05$  in the Mann-Whitney test after Benjamini-Hochberg correction.

To minimize the effect of the initial wound size variance, for each patient the wound size measured at subsequent visits was divided by the wound size during the application of treatment, yielding a relative wound size (wound size quotient). The quotients on day 7, day 14, day 21, and day 49 are named Q1, Q2, Q3 and Q4, respectively. These new variables were used in several statistical analyses:

- To compare the healing rate in both groups, the Wilcoxon-Mann-Whitney test was used;
- Exponential regression was used to determine the half-time of wound surface decline;

- A Spearman correlation was used to evaluate the relationship between the rate of healing and upregulated protein expression. The correlations were calculated between the relative wound size and a measure of increase in protein expression. This measure was defined as the difference in the logarithm of the protein intensities from the mass spectrometry measurements. The measurements were performed in weeks 0 and 1.

To test whether the number of adverse events was different in the two groups, a two-sample Poisson test was used.

## 5. Conclusions

Successful ADSC-related improvement of wound healing correlated with the molecular profile, shedding light on the principles of cell-based therapy for DFU.

The encouraging clinical outcomes warrant further investigation of ADSC-based DFU treatment.

The accessibility of the material of treated wound sites at successive observation timepoints, as done here, presents a unique opportunity for further research on controlling the healing process and understanding the delivered cell-related pathway.

Given the state of the art so far, where the mode of action of cells applied for therapy has been elucidated mainly by in vitro studies and animal experiments, such a possibility might be a fundamental step toward realizing cell-based therapies for humans.

**Supplementary Materials:** The supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms24021472/s1>. References are cited in [33–69].

**Author Contributions:** Conceptualization, B.M.-R., I.S.-G., D.C., R.P., T.G. and M.L.-S.; methodology, B.M.-R., I.S.-G., D.C., S.R., R.P., P.G., M.K., J.Z., M.M., T.G., M.N. and M.L.-S.; validation, B.M.-R., I.S.-G., D.C., R.P., T.G., S.R., M.N. and M.L.-S.; formal analysis, D.C., S.R., R.P., P.G., M.K., J.Z. and B.P.; investigation, B.M.-R., I.S.-G., S.R., M.M., D.S., B.P. and M.N.; resources, B.M.-R., I.S.-G., M.M., D.S., M.N., L.C. and M.L.-S.; data curation, D.C., R.P., P.G., M.K., M.N., L.C. and M.L.-S.; writing—original draft preparation, B.M.-R., I.S.-G., D.C., S.R., R.P., P.G., M.K., J.Z., M.M., D.S., B.P., T.G. and M.L.-S.; writing—review & editing, B.M.-R., I.S.-G., D.C., S.R., T.G., L.C. and M.L.-S.; visualization, D.C., S.R., P.G., M.M., B.P. and T.G.; supervision, B.M.-R., I.S.-G., R.P., L.C. and M.L.-S.; project administration, L.C. and M.L.-S.; funding acquisition, M.L.-S. All authors have read and agreed to the published version of the manuscript.

**Funding:** The research was funded by the National Center for Research and Development, grant number STRATEGMED2/267976/13/NCBR/2015 and by the Medical Research Agency, Poland, from state budget funds, grant number 2021/ABM/03/00037.

**Institutional Review Board Statement:** This study was conducted in accordance with the Declaration of Helsinki, and approved by the Bioethics Committee of Medical University of Warsaw, protocol code: KB/128/2019 24.06.2019 and KB/3/A2021 18.01.2021.

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** All data generated or analyzed during this study are included in the published article and its online supplementary files.

**Acknowledgments:** The study was carried out with the use of Center for Preclinical Research, Medical University of Warsaw, Warsaw, Poland infrastructure, financed by the European Union through the European Regional Development Fund within operational program Innovative Economy for 2007–2013.

**Conflicts of Interest:** The authors declare that they have no conflict of interest.

## References

1. Bandyk, D.F. The Diabetic Foot: Pathophysiology, Evaluation, and Treatment. *Semin. Vasc. Surg.* **2018**, *31*, 43–48. [CrossRef] [PubMed]
2. Tran, H.Q.; Shahriar, S.S.; Yan, Z.; Xie, J. Recent Advances in Functional Wound Dressings. *Adv. Wound Care* **2022**. [CrossRef] [PubMed]
3. FrykbergRobert, G. Challenges in the Treatment of Chronic Wounds. *Adv. Wound Care* **2015**, *4*, 560–582. [CrossRef]



4. Marfia, G.; Navone, S.E.; Di Vito, C.; Ughi, N.; Tabano, S.; Miozzo, M.; Tremolada, C.; Bolla, G.; Crotti, C.; Ingegnoli, F.; et al. Mesenchymal Stem Cells: Potential for Therapy and Treatment of Chronic Non-Healing Skin Wounds. *Organogenesis* **2015**, *11*, 183–206. [\[CrossRef\]](#) [\[PubMed\]](#)
5. Friedenstein, A.J. Precursor Cells of Mechanocytes. *Int. Rev. Cytol.* **1976**, *47*, 327–359. [\[PubMed\]](#)
6. Friedenstein, A.J.; Chailakhyan, R.K.; Gerasimov, U.V. Bone Marrow Osteogenic Stem Cells: In Vitro Cultivation and Transplantation in Diffusion Chambers. *Cell Prolif.* **1987**, *20*, 263–272. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Marquez-Curtis, L.A.; Janowska-Wieczorek, A.; McGann, L.E.; Elliott, J.A. Mesenchymal Stromal Cells Derived from Various Tissues: Biological, Clinical and Cryopreservation Aspects. *Cryobiology* **2015**, *71*, 181–197. [\[CrossRef\]](#)
8. Mazini, L.; Rochette, L.; Admou, B.; Amal, S.; Malka, G. Hopes and Limits of Adipose-Derived Stem Cells (Adscs) and Mesenchymal Stem Cells (Mscs) in Wound Healing. *Int. J. Mol. Sci.* **2020**, *21*, 1306. [\[CrossRef\]](#)
9. Hassanshahi, A.; Hassanshahi, M.; Khabbazi, S.; Hosseini-Khah, Z.; Peymanfar, Y.; Ghalamkari, S.; Su, Y.; Xian, C.J. Adipose-Derived Stem Cells for Wound Healing. *J. Cell. Physiol.* **2018**, *234*, 7903–7914. [\[CrossRef\]](#)
10. Ceccarelli, S.; Pontecorvi, P.; Anastasiadou, E.; Napoli, C.; Marchese, C. Immunomodulatory Effect of Adipose-Derived Stem Cells: The Cutting Edge of Clinical Application. *Front. Cell Dev. Biol.* **2020**, *8*, 236. [\[CrossRef\]](#)
11. Liu, Y.; Holmes, C. Tissue Regeneration Capacity of Extracellular Vesicles Isolated from Bone Marrow-Derived and Adipose-Derived Mesenchymal Stromal/Stem Cells. *Front. Cell Dev. Biol.* **2021**, *9*, 648098. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Waldner, M.; Zhang, W.; James, I.B.; Allbright, K.; Havis, E.; Biley, J.M.; Almadori, A.; Schweizer, R.; Plock, J.A.; Washington, K.M.; et al. Characteristics and Immunomodulating Functions of Adipose-Derived and Bone Marrow-Derived Mesenchymal Stem Cells across Defined Human Leukocyte Antigen Barriers. *Front. Immunol.* **2018**, *9*, 1642. [\[CrossRef\]](#)
13. Gadelkarim, M.; Abushouk, A.I.; Ghanem, E.; Hamaad, A.M.; Saad, A.M.; Abdel-Daim, M.M. Adipose-Derived Stem Cells: Effectiveness and Advances in Delivery in Diabetic Wound Healing. *Biomed. Pharmacother.* **2018**, *107*, 625–633. [\[CrossRef\]](#)
14. Moon, K.-C.; Suh, H.-S.; Kim, K.-B.; Han, S.-K.; Young, K.-W.; Lee, J.-W.; Kim, M.-H. Potential of Allogeneic Adipose-Derived Stem Cell-Hydrogel Complex for Treating Diabetic Foot Ulcers. *Diabetes* **2019**, *68*, 837–846. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Liu, H.; Liu, H.; Deng, X.; Chen, M.; Han, X.; Yan, W.; Wang, N. CXCR4 antagonist delivery on decellularized skin scaffold facilitates impaired wound healing in diabetic mice by increasing expression of SDF-1 and enhancing migration of CXCR4-positive cells. *Wound Repair Regen.* **2017**, *25*, 652–664. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Xu, J.; Hu, J.; Idlett-Ali, S.; Zhang, L.; Caples, K.; Peddibhotla, S.; Reeves, M.; Zgheib, C.; Malany, S.; Liechty, K.W. Discovery of Small Molecule Activators of Chemokine Receptor CXCR4 That Improve Diabetic Wound Healing. *Int. J. Mol. Sci.* **2022**, *23*, 2196. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Griss, J.; Viteri, G.; Sidiropoulos, K.; Nguyen, V.; Fabregat, A.; Hermjakob, H. Reactomega-Efficient Multi-Omics Comparative Pathway Analysis. *Mol. Cell. Proteom.* **2020**, *19*, 2115–2125. [\[CrossRef\]](#)
18. Preda, M.B.; Neculachi, C.A.; Fenyó, L.M.; Vacaru, A.-M.; Publik, M.A.; Simionescu, M.; Burlacu, A. Short Lifespan of Syngeneic Transplanted Msc Is a Consequence of in Vivo Apoptosis and Immune Cell Recruitment in Mice. *Cell Death Dis.* **2021**, *12*, 566. [\[CrossRef\]](#)
19. Wang, Q.-L.; Wang, H.-J.; Li, Z.-H.; Wang, Y.-L.; Wu, X.-P.; Tan, Y.-Z. Mesenchymal Stem Cell-Loaded Cardiac Patch Promotes Epicardial Activation and Repair of the Infarcted Myocardium. *J. Cell. Mol. Med.* **2017**, *21*, 1751–1766. [\[CrossRef\]](#)
20. Ding, Z.; Gau, D.; Deasy, B.; Wells, A.; Roy, P. Both Actin and Polyproline Interactions of Profilin-1 Are Required for Migration, Invasion and Capillary Morphogenesis of Vascular Endothelial Cells. *Exp. Cell Res.* **2009**, *315*, 2963–2973. [\[CrossRef\]](#)
21. González-Pecchi, V.; Valdés, S.; Pons, V.; Honorato, P.; Martínez, L.O.; Lamperti, L.; Aguayo, C.; Radojkovic, C. Apolipoprotein a-I Enhances Proliferation of Human Endothelial Progenitor Cells and Promotes Angiogenesis through the Cell Surface Atp Synthase. *Microvasc. Res.* **2015**, *98*, 9–15. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Nakano, T.; Goto, S.; Takaoka, Y.; Tseng, H.; Fujimura, T.; Kawamoto, S.; Ono, K.; Chen, C. A Novel Moonlight Function of Glyceraldehyde-3-Phosphate Dehydrogenase (Gapdh) for Immunomodulation. *Biofactors* **2017**, *44*, 597–608. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Park, Y.S.; Uddin, J.; Piao, L.; Hwang, I.; Lee, J.H.; Ha, H. Novel Role of Endogenous Catalase in Macrophage Polarization in Adipose Tissue. *Mediat. Inflamm.* **2016**, *2016*, 8675905. [\[CrossRef\]](#)
24. Karantza, V. Keratins in Health and Cancer: More Than Mere Epithelial Cell Markers. *Oncogene* **2011**, *30*, 127–138. [\[CrossRef\]](#)
25. Hamill, K.J.; Hiroyasu, S.; Colburn, Z.T.; Ventrella, R.V.; Hopkinson, S.B.; Skalli, O.; Jones, J.C. Alpha Actinin-1 Regulates Cell-Matrix Adhesion Organization in Keratinocytes: Consequences for Skin Cell Motility. *J. Invest. Dermatol.* **2015**, *135*, 1043–1052. [\[CrossRef\]](#)
26. Johnson, T.R.; Gómez, B.I.; McIntyre, M.K.; Dubick, M.A.; Christy, R.J.; Nicholson, S.E.; Burmeister, D.M. The Cutaneous Microbiome and Wounds: New Molecular Targets to Promote Wound Healing. *Int. J. Mol. Sci.* **2018**, *19*, 2699. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Burdukiewicz, M.; Sidorczuk, K.; Rafacz, D.; Pietluch, F.; Chilimoniuk, J.; Rödiger, S.; Gagat, P. Proteomic Screening for Prediction and Design of Antimicrobial Peptides with Ampgram. *Int. J. Mol. Sci.* **2020**, *21*, 4310. [\[CrossRef\]](#)
28. Mahlapuu, M.; Sidorowicz, A.; Mikosinski, J.; Krzyżanowski, M.; Orleanski, J.; Twardowska-Sauchka, K.; Nykaza, A.; Dyaczynski, M.; Belz-Lagoda, B.; Dziwiszek, G.; et al. Evaluation of LI-37 in Healing of Hard-to-Heal Venous Leg Ulcers: A Multicentric Prospective Randomized Placebo-Controlled Clinical Trial. *Wound Repair Regen.* **2021**, *6*, 938–950. [\[CrossRef\]](#)
29. Mangoni, M.L.; McDermott, A.M.; Zasloff, M. Antimicrobial Peptides and Wound Healing: Biological and Therapeutic Considerations. *Exp. Dermatol.* **2016**, *25*, 167–173. [\[CrossRef\]](#)
30. Mildmay-White, A.; Khan, W. Cell Surface Markers on Adipose-Derived Stem Cells: A Systematic Review. *Curr. Stem Cell Res. Ther.* **2017**, *12*, 484–492. [\[CrossRef\]](#)

31. Wessel, D.; Flügel, U.I. A Method for the Quantitative Recovery of Protein in Dilute Solution in the Presence of Detergents and Lipids. *Anal. Biochem.* **1984**, *138*, 141–143. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Kuzniowska, B.; Cysewski, D.; Wasilewski, M.; Sakowska, P.; Milek, J.; Kulinski, T.M.; Winiarski, M.; Kozieliwicz, P.; Knapka, E.; Dadlez, M.; et al. Mitochondrial Protein Biogenesis in the Synapse Is Supported by Local Translation. *EMBO Rep.* **2020**, *21*, e48882. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Basu, A.; Munir, S.; Mulaw, M.A.; Singh, K.; Crisan, D.; Sindrilaru, A.; Treiber, N.; Wlaschek, M.; Huber-Lang, M.; Gebhard, F.; et al. A Novel S100a8/A9 Induced Fingerprint of Mesenchymal Stem Cells Associated with Enhanced Wound Healing. *Sci. Rep.* **2018**, *8*, 6205. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Sroussi, H.Y.; Köhler, G.A.; Agabian, N.; Villines, D.; Palefsky, J.M. Substitution of Methionine 63 or 83 in S100a9 and Cysteine 42 in S100a8 Abrogate the Antifungal Activities of S100a8/A9: Potential Role for Oxidative Regulation. *FEMS Immunol. Med. Microbiol.* **2009**, *55*, 55–61. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Kehl-Fie, T.E.; Chitayat, S.; Hood, M.I.; Damo, S.; Restrepo, N.; Garcia, C.; Munro, K.A.; Chazin, W.J.; Skaar, E.P. Nutrient Metal Sequestration by Calprotectin Inhibits Bacterial Superoxide Defense, Enhancing Neutrophil Killing of Staphylococcus Aureus. *Cell Host Microbe* **2011**, *10*, 158–164. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Shabani, F.; Farasat, A.; Mahdavi, M.; Gheibi, N. Calprotectin (S100a8/S100a9): A Key Protein between Inflammation and Cancer. *Inflamm. Res.* **2018**, *67*, 801–812. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Jin, M.-H.; Yu, N.-N.; Jin, Y.-H.; Mao, Y.-Y.; Feng, L.; Liu, Y.; Wang, A.-G.; Sun, H.-N.; Kwon, T.; Han, Y.-H. Peroxiredoxin Ii with Dermal Mesenchymal Stem Cells Accelerates Wound Healing. *Aging* **2021**, *13*, 13926. [\[CrossRef\]](#)
38. Takayama, Y.; Aoki, R. Roles of Lactoferrin on Skin Wound Healing. *Biochem. Cell Biol.* **2011**, *90*, 497–503. [\[CrossRef\]](#)
39. Lu, J.; Guevara, M.A.; Francis, J.D.; Spicer, S.K.; Moore, R.E.; Chambers, S.A.; Craft, K.M.; Manning, S.D.; Townsend, S.D.; Gaddy, J.A. Analysis of Susceptibility to the Antimicrobial and Anti-Biofilm Activity of Human Milk Lactoferrin in Clinical Strains of Streptococcus Agalactiae with Diverse Capsular and Sequence Types. *Front. Cell. Infect. Microbiol.* **2021**, *11*, 740872. [\[CrossRef\]](#)
40. Gupta, A.K.; Parasar, D.; Sagar, A.; Choudhary, V.; Chopra, B.S.; Garg, R.; Ashish; Khatri, N. Analgesic and Anti-Inflammatory Properties of Gelsolin in Acetic Acid Induced Writhing, Tail Immersion and Carrageenan Induced Paw Edema in Mice. *PLoS ONE* **2015**, *10*, e0135558. [\[CrossRef\]](#)
41. Witke, W.; Sharpe, A.H.; Hartwig, J.H.; Azuma, T.; Stossel, T.P.; Kwiatkowski, D.J. Hemostatic, Inflammatory, and Fibroblast Responses Are Blunted in Mice Lacking Gelsolin. *Cell* **1995**, *81*, 41–51. [\[CrossRef\]](#)
42. Yang, Y.-W.; Zhang, C.-N.; Cao, Y.-J.; Qu, Y.-X.; Li, T.-Y.; Yang, T.-G.; Geng, D.; Sun, Y.-K. Bidirectional Regulation of I-Type Lysozyme on Cutaneous Wound Healing. *Biomed. Pharmacother.* **2020**, *131*, 110700. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Schröder, J.M.; Harder, J. Antimicrobial Skin Peptides and Proteins. *Cell. Mol. Life Sci. CMLS* **2006**, *63*, 469–486. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Singer, A.J.; Clark, R.A.F. Cutaneous Wound Healing. *N. Engl. J. Med.* **1999**, *341*, 738–746. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Kaijzel, E.L.; Koolwijk, P.; VAN Erck, M.G.M.; VAN Hinsbergh, V.W.M.; DE Maat, M.P.M. Molecular Weight Fibrinogen Variants Determine Angiogenesis Rate in a Fibrin Matrix in Vitro and in vivo. *J. Thromb. Haemost.* **2006**, *4*, 1975–1981. [\[CrossRef\]](#)
46. Duong, L.; Gross, S.P.; Sityaporn, A. A Novel Antibacterial Strategy: Histone and Antimicrobial Peptide Synergy. *Microb. Cell* **2020**, *7*, 309. [\[CrossRef\]](#)
47. Brookes, J.D.L.; Jaya, J.S.; Tran, H.; Vaska, A.; Werner-Gibbings, K.; D'Mello, A.C.; Wong, J.; Lemoh, C.N.; Saunderson, A.C.; Yui, M.K. Broad-Ranging Nutritional Deficiencies Predict Amputation in Diabetic Foot Ulcers. *Int. J. Low. Extrem. Wounds* **2020**, *19*, 27–33. [\[CrossRef\]](#)
48. Mockenhaupt, M.; Viboud, C.; Dunant, A.; Naldi, L.; Halevy, S.; Bavinck, J.N.B.; Sidoroff, A.; Schneck, J.; Roujeau, J.C.; Flahault, A. Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis: Assessment of Medication Risks with Emphasis on Recently Marketed Drugs. The Euroscar-Study. *J. Investig. Dermatol.* **2008**, *128*, 35–44. [\[CrossRef\]](#) [\[PubMed\]](#)
49. Suri, B.; Verma, N.; Schmidtchen, A. Toll-Like Receptor 3 Agonist, Polyinosinic-Polycytidylic Acid, Upregulates Carbonic Anhydrase Ii in Human Keratinocytes. *Acta Dermato-Venerol.* **2018**, *98*, 762–765. [\[CrossRef\]](#)
50. Pechter, P.M.; Gil, J.; Valdes, J.; Tomic-Canic, M.; Pastar, I.; Stojadinovic, O.; Kirsner, R.S.; Davis, S.C. Keratin Dressings Speed Epithelialization of Deep Partial-Thickness Wounds. *Wound Repair Regen.* **2012**, *20*, 236–242. [\[CrossRef\]](#)
51. Roth, W.; Hatzfeld, M.; Magin, T.M. Targeting the Palm: A Leap Forward toward Treatment of Keratin Disorders. *J. Investig. Dermatol.* **2012**, *132*, 1541–1542. [\[CrossRef\]](#) [\[PubMed\]](#)
52. Moll, R.; Divo, M.; Langbein, L. The Human Keratins: Biology and Pathology. *Histochem. Cell Biol.* **2008**, *129*, 705–733. [\[CrossRef\]](#) [\[PubMed\]](#)
53. Nielsen, M.M.K.; Aryal, E.; Safari, E.; Mojsoska, B.; Jenssen, H.; Prabhala, B.K. Current State of Slc and Abc Transporters in the Skin and Their Relation to Sweat Metabolites and Skin Diseases. *Proteomes* **2021**, *9*, 23. [\[CrossRef\]](#)
54. D'Alessandro, S.; Magnavacca, A.; Perego, F.; Fumagalli, M.; Sangiovanni, E.; Prato, M.; Dell'Agli, M.; Basilico, N. Effect of Hypoxia on Gene Expression in Cell Populations Involved in Wound Healing. *BioMed Res. Int.* **2019**, *2019*, 2626374. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Edwards, J.V.; Howley, P.; Davis, R.; Mashchak, A.; Goheen, S.C. Protease Inhibition by Oleic Acid Transfer from Chronic Wound Dressings to Albumin. *Int. J. Pharm.* **2007**, *340*, 42–51. [\[CrossRef\]](#)
56. Bangalore, N.; Travis, J.; Onunka, V.C.; Pohl, J.; Shafer, W.M. Identification of the Primary Antimicrobial Domains in Human Neutrophil Cathepsin G. *J. Biol. Chem.* **1990**, *265*, 13584–13588. [\[CrossRef\]](#)
57. Cazander, G.; Jukema, G.N.; Nibbering, P.H. Complement Activation and Inhibition in Wound Healing. *Clin. Dev. Immunol.* **2012**, *2012*, 534291. [\[CrossRef\]](#)



58. Rafail, S.; Kourtzelis, I.; Foukas, P.G.; Markiewski, M.M.; DeAngelis, R.A.; Guariento, M.; Ricklin, D.; Grice, E.; Lambris, J.D. Complement Deficiency Promotes Cutaneous Wound Healing in Mice. *J. Immunol.* **2014**, *194*, 1285–1291. [\[CrossRef\]](#)
59. Ganeshan, K.; Chawla, A. Metabolic Regulation of Immune Responses. *Annu. Rev. Immunol.* **2014**, *32*, 609. [\[CrossRef\]](#)
60. Im, M.J.; Hoopes, J.E. Energy Metabolism in Healing Skin Wounds. *J. Surg. Res.* **1970**, *10*, 459–464. [\[CrossRef\]](#)
61. Donaldson, D.J.; Mahan, J.T. Fibrinogen and Fibronectin as Substrates for Epidermal Cell Migration During Wound Closure. *J. Cell Sci.* **1983**, *62*, 117–127. [\[CrossRef\]](#) [\[PubMed\]](#)
62. Martin, P. Wound Healing—Aiming for Perfect Skin Regeneration. *Science* **1997**, *276*, 75–81. [\[CrossRef\]](#) [\[PubMed\]](#)
63. Maione, A.G.; Smith, A.; Kashpur, O.; Yanez, V.; Knight, E.; Mooney, D.J.; Veves, A.; Tomic-Canic, M.; Garlick, J.A. Altered Ecm Deposition by Diabetic Foot Ulcer-Derived Fibroblasts Implicates Fibronectin in Chronic Wound Repair. *Wound Repair Regen.* **2016**, *24*, 630–643. [\[CrossRef\]](#)
64. Dimayuga, P.; Zhu, J.; Oguchi, S.; Chyu, K.Y.; Xu, X.O.H.; Yano, J.; Shah, P.K.; Nilsson, J.; Cercek, B. Reconstituted Hdl Containing Human Apolipoprotein a-1 Reduces Vcam-1 Expression and Neointima Formation Following Periadventitial Cuff-Induced Carotid Injury in Apoe Null Mice. *Biochem. Biophys. Res. Commun.* **1999**, *264*, 465–468. [\[CrossRef\]](#) [\[PubMed\]](#)
65. Vowinkel, T.; Mori, M.; Kriegelstein, C.F.; Russell, J.; Saijo, F.; Bharwani, S.; Turnage, R.H.; Davidson, W.; Tso, P.; Granger, D.N.; et al. Apolipoprotein a-Iv Inhibits Experimental Colitis. *J. Clin. Investig.* **2004**, *114*, 260–269. [\[CrossRef\]](#)
66. Xu, X.R.; Wang, Y.; Adili, R.; Ju, L.; Spring, C.M.; Jin, J.W.; Yang, H.; Neves, M.A.; Chen, P.; Yang, Y.; et al. Apolipoprotein a-Iv Binds Aiiβ3 Integrin and Inhibits Thrombosis. *Nat. Commun.* **2018**, *9*, 3608. [\[CrossRef\]](#)
67. Karahan, A.; AAbbasoglu, A.; Işık, S.A.; Çevik, B.; Saltan, Ç.; Elbaş, N.Ö.; Yalılı, A. Factors Affecting Wound Healing in Individuals with Pressure Ulcers: A Retrospective Study. *Ostomy/Wound Manag.* **2018**, *64*, 32–39. [\[CrossRef\]](#)
68. Płoski, R.; Pollak, A.; Müller, S.; Franaszczyk, M.; Michalak, E.; Kosinska, J.; Stawinski, P.; Spiewak, M.; Seggewiss, H.; Bilinska, Z.T. Does P. Q247x in Trim63 Cause Human Hypertrophic Cardiomyopathy? *Circ. Res.* **2014**, *114*, e2–e5. [\[CrossRef\]](#)
69. Karczewski, K.J.; Francioli, L.C.; Tiao, G.; Cummings, B.B.; Alfoldi, J.; Wang, Q.; Collins, R.L.; Laricchia, K.M.; Ganna, A.; Birnbaum, D.P.; et al. The Mutational Constraint Spectrum Quantified from Variation in 141,456 Humans. *Nature* **2020**, *581*, 434–443. [\[CrossRef\]](#)

**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.



### 6.3. The potential of mesenchymal stem/stromal cells in diabetic wounds and future directions for research and therapy—is it time for use in everyday practice?



#### Review

## The Potential of Mesenchymal Stem/Stromal Cells in Diabetic Wounds and Future Directions for Research and Therapy—Is It Time for Use in Everyday Practice?

Damian Sierko <sup>1,2</sup>, Ilona Szablowska-Gadomska <sup>3</sup>, Anna Nowak-Szwed <sup>1,2</sup>, Stefan Rudziński <sup>3</sup>, Maksymilian Gofron <sup>4</sup>, Przemysław Zygmunciak <sup>5</sup>, Małgorzata Lewandowska-Szumiel <sup>3,6</sup>, Wojciech Stanisław Zgliczyński <sup>5</sup>, Leszek Czupryniak <sup>1</sup> and Beata Mrozikiewicz-Rakowska <sup>5,\*</sup>

<sup>1</sup> Department of Diabetology and Internal Diseases, Medical University of Warsaw, 02-097 Warsaw, Poland; damiansierko7@gmail.com (D.S.); anna.nowak@wum.edu.pl (A.N.-S.); bigosik@poczta.onet.pl (L.C.)

<sup>2</sup> Doctoral School, Medical University of Warsaw, 02-091 Warsaw, Poland

<sup>3</sup> Laboratory for Cell Research and Application, Medical University of Warsaw, 02-097 Warsaw, Poland; ilona.szablowska-gadomska@wum.edu.pl (I.S.-G.); stefan.rudzinski@wum.edu.pl (S.R.); malgorzata.lewandowska-szumiel@wum.edu.pl (M.L.-S.)

<sup>4</sup> Department of Urology, Municipal Complex Hospital, 42-200 Częstochowa, Poland; max9615@gmail.com

<sup>5</sup> Department of Endocrinology, Centre of Postgraduate Medical Education, Bielanski Hospital, 01-809 Warsaw, Poland; zygmunciakprzemyslaw@gmail.com (P.Z.); zgliczynski.w@gmail.com (W.S.Z.)

<sup>6</sup> Department of Histology and Embryology, Medical University of Warsaw, 02-004 Warsaw, Poland

\* Correspondence: bmrozikiewicz@cmkmp.edu.pl



**Citation:** Sierko, D.; Szablowska-Gadomska, I.; Nowak-Szwed, A.; Rudziński, S.; Gofron, M.; Zygmunciak, P.; Lewandowska-Szumiel, M.; Zgliczyński, W.S.; Czupryniak, L.; Mrozikiewicz-Rakowska, B. The Potential of Mesenchymal Stem/Stromal Cells in Diabetic Wounds and Future Directions for Research and Therapy—Is It Time for Use in Everyday Practice? *Int. J. Mol. Sci.* **2024**, *25*, 12171. <https://doi.org/10.3390/ijms252212171>

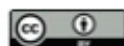
Academic Editor: Young Woo Eom

Received: 5 October 2024

Revised: 30 October 2024

Accepted: 7 November 2024

Published: 13 November 2024



**Copyright:** © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

**Abstract:** The treatment of diabetic wounds is impaired by the intricate nature of diabetes and its associated complications, necessitating novel strategies. The utilization of mesenchymal stem/stromal cells (MSCs) as a therapeutic modality for chronic and recalcitrant wounds in diabetic patients is an active area of investigation aimed at enhancing its therapeutic potential covering tissue regeneration. The threat posed to the patient and their environment by the presence of a diabetic foot ulcer (DFU) is so significant that any additional therapeutic approach that opens new pathways to halt the progression of local changes, which subsequently lead to a generalized inflammatory process, offers a chance to reduce the risk of amputation or even death. This article explores the potential of MSCs in diabetic foot ulcer treatment, examining their mechanisms of action, clinical application challenges, and future directions for research and therapy.

**Keywords:** mesenchymal stem/stromal cells; cell therapy; diabetic foot ulcer; allogenic cell therapy; ATMP

### 1. Introduction

The global incidence of diabetes mellitus (DM) in individuals aged 20–79 years was estimated at 10.5%, encompassing approximately 536.6 million individuals in 2021. This figure is projected to rise to 12.2%, affecting an estimated 783.2 million by 2045 [1]. A prevalent complication of DM, diabetic foot ulcer (DFU), afflicts up to one-third of patients over their lifetime, leading to substantial morbidity characterized by challenging treatment courses and a high recurrence rate.

The management of chronic and recalcitrant wounds in diabetic patients remains a significant challenge in contemporary medicine. The intricacies of wound healing in diabetic patients, compounded by impaired angiogenesis, delayed re-epithelialization, and diminished collagen deposition, contribute to the chronic nature of these wounds [2,3]. In light of the constraints inherent in conventional therapeutic modalities for diabetic wound care, contemporary research has increasingly pivoted toward exploring the potential of mesenchymal stem/stromal cells (MSCs) as a novel therapeutic approach in this domain. MSCs are extensively recognized for their crucial role in immunomodulation, hematopoiesis, and

tissue repair. Due to these distinctive characteristics, they have gained rapid recognition as a promising therapeutic agent in the field of regenerative medicine [4,5]. MSCs are multipotent cells capable of differentiating into adipocytes, osteoblasts, and chondrocytes. They are identified by positive expressions of CD73, CD90, and CD105, and the absence of hematopoietic markers like CD34 and CD45. MSCs can be derived from various tissues, including bone marrow and adipose tissue, and exhibit heterogeneity based on their origin. In clinical contexts, pure MSCs refer to isolated, well-characterized cell populations that meet specific phenotypic and functional criteria. In contrast, MSCs in the stromal vascular fraction (SVF) are part of a mixed cell population, containing other cell types like endothelial and immune cells. This distinction is critical for interpreting clinical outcomes, as pure MSCs offer targeted effects, whereas SVF-derived therapies involve multiple cell interactions, potentially complicating the understanding of therapeutic efficacy [6,7]. MSCs, particularly those derived from sources like umbilical cords and adipose tissue, have shown promise in enhancing wound healing through differentiation and angiogenesis [3,8]. In the context of diabetic foot ulcers (DFUs), MSCs exhibit the capacity to modulate various critical processes essential for efficacious wound healing. As demonstrated by randomized clinical trials, stem cells are capable of improving the treatment of DFU, reducing its cost, and additionally decreasing the frequency of hospital admissions for patients with this complication [9–12]. These processes include the modulation of inflammatory responses, the synthesis of the extracellular matrix, the facilitation of keratinocyte migration, and the promotion of angiogenesis [13–15]. In pursuit of augmenting the therapeutic efficacy of MSCs, several innovative methodologies are being employed. These include preconditioning of MSCs, genetic modification, the application of combination therapies, and the utilization of exosome-based approaches [16]. For instance, recent advancements in elucidating the role of MSC-derived extracellular vesicles (EVs) have unveiled novel therapeutic pathways. These developments promise substantial benefits in the realms of tissue repair and regeneration, particularly in the treatment of diabetic wounds [17].

Despite significant progress in delineating the pathophysiology of DFUs and associated cellular and molecular mechanisms, there remains a notable deficit in efficacious treatment modalities. Present therapeutic approaches are often ineffectual in expeditiously healing deep, chronic wounds, particularly those complicated by microvascular obstruction. This challenge is further exacerbated by the frequent recurrence of DFUs, coupled with their correlation to elevated mortality rates and substantial healthcare expenditures. The current evidence underpinning much of the routine clinical management of DFUs is, regrettably, limited. This paucity of robust evidence underscores the imperative need for enhanced clinical trials and systematic evaluations of routine care outcomes, particularly across diverse health economies [18,19].

This manuscript endeavors to investigate the potential applications of MSCs in the therapeutic management of DFUs. It aims to elucidate the underlying mechanisms of action of MSCs, delineate the challenges associated with their clinical application, and forecast future trajectories for both research and therapeutic interventions in this domain.

## 2. Pathophysiology of Impaired Wound Healing in Diabetes Mellitus

Impaired wound healing in diabetes results from several overlapping processes, including endotheliopathy, neuropathy, and immunopathy.

### 2.1. Endotheliopathy

Endotheliopathy plays a critical role in the dysfunction of wound healing observed in patients with diabetes mellitus, acting as a fundamental mechanism that underpins the complex interplay between vascular, neuropathic, and metabolic disturbances characteristic of this condition. The pathological changes initiated by endothelial dysfunction in diabetes include both microvascular and macrovascular alterations, which collectively impair blood flow, reduce nutrient and oxygen delivery to the wound site, and hinder the removal of metabolic waste products [20]. Research underscores that the persistent hyperglycemic



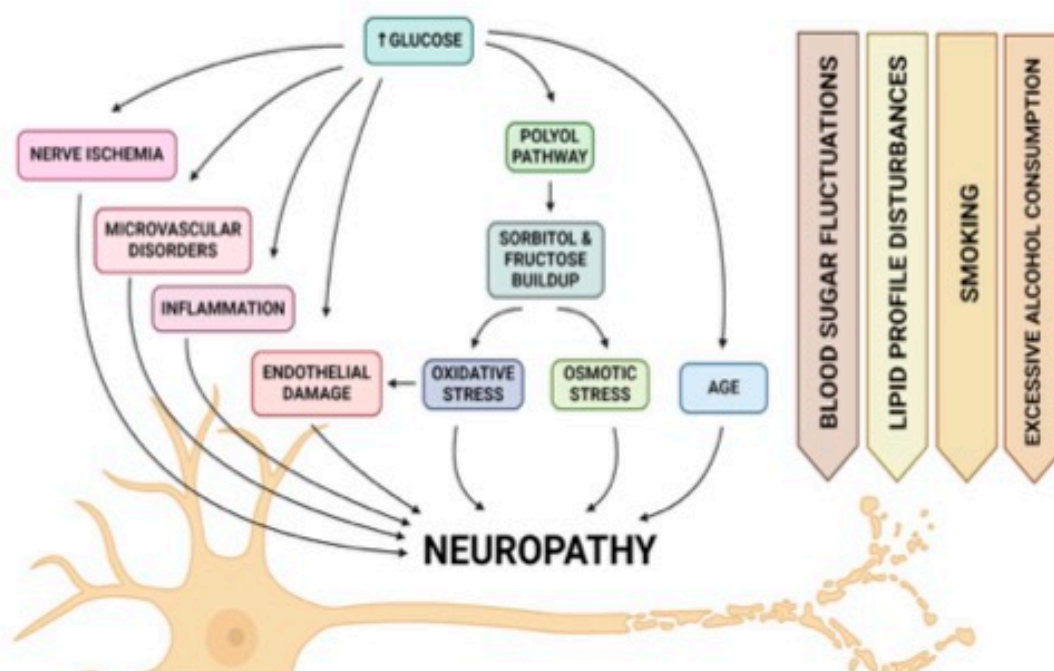
state characteristic of type 2 diabetes mellitus initiates non-enzymatic glycation reactions with proteins, lipids, and nucleic acids, leading to the formation of advanced glycation end products (AGEs). These AGEs are central to the progression of diabetic complications, contributing to oxidative stress and inflammation through various mechanisms, including the activation of the receptor for AGEs pathway. This process, in turn, exacerbates endothelial damage and impairs the natural angiogenic response necessary for tissue repair and vascular health [21–23]. Furthermore, hyperglycemia-induced metabolic derangements in endothelial cells, as evidenced by experimental models, lead to increased endothelial cell layer permeability and decreased capillary density. These changes underline the vascular dysfunction integral to diabetic cardiomyopathy, emphasizing the role of chronic hyperglycemia in inducing oxidative stress within diabetic endothelial cells [24]. This understanding is pivotal for developing targeted therapeutic interventions aimed at mitigating these vascular complications.

Research indicates that in diabetic patients, impaired immune response significantly hampers wound healing. Diabetic foot ulcers show a lack of necessary immune cell recruitment for normal healing processes. This deficiency points to potential targets for future therapeutic interventions, highlighting the importance of understanding how the healing process is disrupted in diabetic wounds to design more effective treatments [25]. Collectively, these factors underscore the critical role of endotheliopathy in the multifaceted process of wound healing dysfunction in diabetes mellitus, highlighting the need for targeted therapeutic strategies to mitigate vascular complications and enhance wound repair. Recent studies suggest MSC-based therapy may have a positive effect on the process of endotheliopathy by reducing endothelial cell damage and inflammation, thus improving vascular function in diabetic patients [26].

## 2.2. Neuropathy

Diabetic neuropathy (DN) is a prevalent and debilitating complication of diabetes mellitus, affecting up to 51% of patients [27]. Recent studies from 2021 onwards continue to support the understanding that chronic hyperglycaemia, activation of the polyol pathway, increased oxidative stress, and the formation of advanced glycation end products (AGEs) are central to the pathogenesis of diabetic neuropathy. Research provides further insights into these contributing factors. The activation of the polyol pathway, a consequence of persistent hyperglycaemia, contributes significantly to nerve damage through the accumulation of sorbitol and fructose, leading to osmotic and oxidative stress within nerve cells [28,29]. Oxidative stress, heightened by chronic hyperglycaemia and acute glucose fluctuations, plays a significant role in endothelial dysfunction and diabetic neuropathy, indicating its role in the deterioration of nerve function [30,31]. Additionally, the formation of AGEs through non-enzymatic reactions between proteins and reducing sugars contributes to the structural and functional alterations of tissues, exacerbating neuropathic conditions in diabetes [32,33]. These above-mentioned mechanisms collectively underscore the complex nature of diabetic neuropathy's development, highlighting the importance of controlling hyperglycaemia and addressing oxidative stress as part of comprehensive management strategies to mitigate the progression of this debilitating complication (Figure 1). In addition to hyperglycaemia, factors including fluctuations in blood sugar levels, disturbances in lipid profiles, smoking, and excessive alcohol consumption can also affect the presence and intensity of neuropathy symptoms [34]. Damage to the vasa nervorum, the blood vessels that nourish diverse nerve fibers including C-type and delta fibers vital for nervous system functions, is primarily linked to the hindered healing of wounds in diabetes mellitus, particularly recurring issues in diabetic foot ulcers [35]. Recent research has shown that MSC-based therapy can significantly influence neuropathy by promoting nerve regeneration and reducing inflammation. MSCs release exosomes that carry proteins, microRNAs, and other factors, which can suppress inflammatory pathways and enhance nerve repair in diabetic neuropathy. These therapeutic effects have been observed in both animal models and preclinical studies, where MSCs improved nerve function and reduced

neuropathic pain, suggesting a promising avenue for treating peripheral neuropathy in diabetic patients [36].



**Figure 1.** Illustration of pathophysiological factors that contribute to the development of neuropathy in diabetic foot syndrome.

### 2.3. Immunopathy

Diabetic immunopathy refers to the complex alterations in the immune system resulting from diabetes mellitus, contributing to a wide array of complications that significantly impact patient morbidity and mortality. The pathophysiology of diabetic immunopathy is multifaceted, involving again hyperglycemia-induced oxidative stress, chronic inflammation, and immune dysregulation, which collectively predispose individuals to infections, impaired wound healing, and accelerated atherosclerosis. Hyperglycemia plays a central role in diabetic immunopathy by promoting oxidative stress and the production of advanced glycation end products (AGEs) [33]. These processes lead to endothelial dysfunction and a pro-inflammatory state, characterized by increased circulating levels of inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) [37]. Chronic inflammation significantly contributes to the dysregulation of both innate and adaptive immunity, impairing neutrophil function and reducing the proliferative response of lymphocytes, thereby compromising the body's ability to combat infections and heal wounds effectively [38–40]. In another study, proteomic analysis demonstrated the presence of proteins that may be responsible for mitigating inflammatory processes in patients with diabetic foot syndrome undergoing allogeneic MSC therapy [26]. The alteration of the immune system in diabetic individuals also exacerbates the severity of viral infections, such as SARS-CoV-2, dengue, and influenza, compared to healthy individuals. The dysregulated immune response in diabetes leads to heightened susceptibility and escalated disease severity, emphasizing the critical need for effective management of diabetes to mitigate these adverse outcomes. Moreover, diabetic immunopathy contributes to the pathogenesis of microvascular and macrovascular complications. The immune-mediated inflammatory processes accelerate the development of diabetic retinopathy, nephropathy,



and neuropathy, with diabetic nephropathy being particularly influenced by the role of innate immunity in promoting inflammation and tissue fibrosis, ultimately leading to renal failure [41–43]. This illustrates the significant impact of immune dysregulation on the progression of diabetes-related complications.

The underlying pathophysiology of diabetes, including impaired angiogenesis, chronic inflammation, and reduced neovascularization, often compromises wound healing in diabetic foot syndrome [44].

### 3. MSC Role in Wound Healing Phases

MSCs have emerged as a promising therapeutic strategy to enhance wound healing in DFU, leveraging their multifaceted role in the wound healing processes, including inflammation, proliferation, and tissue remodeling [45,46].

#### 3.1. Inflammatory Phase

In diabetic patients, the inflammatory phase of the wound healing process is often prolonged, leading to chronic wound occurrence. MSCs have the potential to react to the inflammatory environment affecting various molecular pathways. In vitro studies have demonstrated that MSCs can reduce the expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$  [47]. They have also shown that MSCs secrete anti-inflammatory cytokines, including interleukin-10 (IL-10) and transforming growth factor-beta (TGF- $\beta$ ), which modulate T-cell responses by suppressing excessive Th1 responses and promoting a shift toward a Th2-type response [48,49]. Another pivotal role of MSCs is the reduction in reactive oxygen species (ROS), which are elevated in diabetic wounds. By regulating ROS levels, MSCs mitigated oxidative stress-induced damage in the murine model, facilitating the resolution of inflammation [50]. Additionally, Cho et al. demonstrated, using an in vitro co-culture system with mouse bone marrow-derived macrophages, that MSCs modulated macrophage polarization, shifting them from a pro-inflammatory (M1) to an anti-inflammatory (M2) phenotype, which supported healing and reduced chronic inflammation [51,52]. All those actions lead to the faster transition from the inflammatory phase to the proliferation phase of wound healing.

#### 3.2. Proliferation Phase

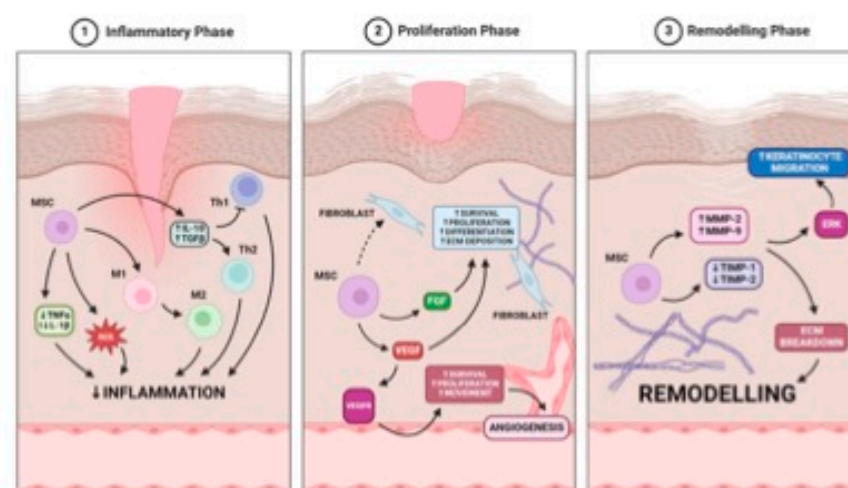
During the proliferation phase, MSCs contribute to tissue formation and repair by promoting the proliferation of fibroblasts, keratinocytes, and endothelial cells, as well as enhancing angiogenesis. Throughout this process, MSCs release paracrine growth factors that facilitate tissue regeneration and promote wound healing [45]. Saheli et al. investigated using MSC-conditioned media (MSC-CM) in enhancing wound healing in diabetic conditions. By using both in vivo diabetic rat models and in vitro high-glucose fibroblast cultures, researchers observed improved wound closure rates, reduced inflammation, better tissue remodeling, and increased vascularization in MSC-CM-treated wounds. Gene analysis showed upregulation of growth factors epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), indicating that MSC-CM promotes fibroblast viability, proliferation, and migration [53]. Yates et al. showed that MSC treatment supports fibroblast function by promoting their survival and movement, which leads to enhanced extracellular matrix (ECM) deposition, boosting overall healing effects in the in vivo murine model [54]. Additionally, Li et al. demonstrated that exosomes derived from MSCs play a crucial role in stimulating fibroblast proliferation and migration, further aiding the wound repair process [55]. At the same time MSCs enhance neovascularization by secretion of growth factors such as vascular endothelial growth factor (VEGF) crucial for new blood vessel formation and wound closure. Wu et al. conducted a study using genetically diabetic db/db mice and discovered that lesions treated with MSCs exhibited significantly increased levels of VEGF, Angiopoietin-1, and keratinocyte-specific proteins. The MSCs enhanced keratinocyte proliferation, stimulated angiogenesis, promoted epithelial regeneration, and accelerated overall wound healing [2,56]. Moreover, in the context of chronic wounds

associated with diabetes, the application of a synthetic three-dimensional collagen scaffold infused with VEGF has been shown to improve blood vessel formation and accelerate wound healing in diabetic murine model [57].

### 3.3. Remodeling Phase

The remodeling phase focuses on the scar's maturation and achieving optimal tensile strength through the rearrangement, breakdown, and renewal of the ECM. In this phase, the density of collagen fibers within the granulation tissue increases as part of the tissue structure's restoration [58]. During the remodeling phase, MSCs modulate the activity of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), balancing the breakdown and synthesis of ECM components and allowing tissue remodeling [59]. Lozito et al. demonstrated this regulatory effect in an in vitro model, showcasing MSCs' capacity to modulate ECM dynamics, essential for effective wound healing and tissue restructuring [60]. Similarly, Li et al. revealed that MSC-CM can significantly modulate ECM remodeling by upregulating mRNA levels of matrix metalloproteinases MMP-2 and MMP-9, while downregulating tissue inhibitors TIMP-1 and TIMP-2, thereby promoting the wound healing remodeling phase [61]. Additionally, MSC-CM activates the Erk signaling pathway, especially in diabetes-mimicking environments, to enhance keratinocyte proliferation and migration [61]. These in vitro findings emphasize MSCs' therapeutic potential in regulating ECM dynamics and support MSC-based therapies to improve tissue repair and regeneration.

Reassuring MSCs offer a promising therapeutic approach for the regeneration of injured tissues in patients with diabetes mellitus, especially with diabetic foot ulcer. Their ability to modulate the inflammatory response, promote tissue proliferation and enhance vasculature regeneration addresses the multifaceted challenges in healing diabetic foot ulcers (Figure 2). However, clinical application requires further research to optimize MSC delivery methods and dosages and understand their mechanisms of action in diabetic foot syndrome.



**Figure 2.** MSCs role in wound healing is based on the modulation of three key phases: the inflammatory phase, where MSCs reduce inflammation via cytokine regulation and macrophage polarization; the proliferation phase, where MSCs enhance fibroblast activity and angiogenesis through VEGF and FGF signaling; and the remodeling phase, where MSCs regulate extracellular matrix breakdown and keratinocyte migration to promote tissue regeneration.

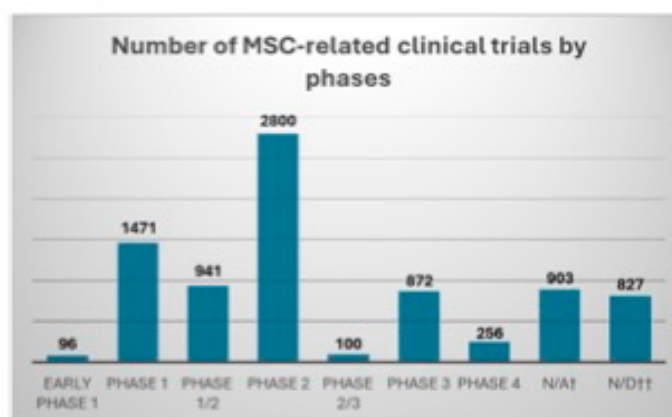


#### 4. The Legal Regulations Regarding Cell-Based Products

Researchers who explore the properties of mesenchymal stem/stromal cells (MSCs) to apply them in clinical practice must navigate a challenging process to prepare these cells for administration to patients. Below, we describe the regulatory issues governing the clinical application of cell-based products. These include compliance with stringent regulations on cell isolation, cultivation, quality control, and safety measures, as well as obtaining the necessary approvals from relevant health authorities to ensure the product's safety and efficacy for therapeutic use. The cell-based medicinal products and related procedures are regulated by law and are considered Advanced Therapy Medicinal Products (ATMP) [62]. Throughout the European Union, there are three main categories of ATMPs. First, somatic-cell therapy medicines (also called somatic Cell Therapy Medical Products- sCTMP) are products containing manipulated cells to change their biological properties or be used for purposes other than their primary function in the human body. The second group of ATMP is called tissue-engineered medicines (TEM) or products (TEP), and it contains cells that are modified in such a way that they can be used to repair, regenerate, or replace diseased or damaged tissues. The third category includes gene therapy medicines (GTM, also called gene therapy medicinal products—GTMP), which are increasingly popular nowadays. These involve introducing laboratory-recombined genes into a patient's body, such as Gene Therapy Medicinal Products (GTMP), for example, Chimeric Antigen Receptor T-cells (CAR-T). There are also combined ATMPs with one or more medical devices, e.g., cells seeded on a scaffold [62–64]. In the EU, the first ATMP product appeared on the market in 2009, and, to date, at least 24 products have been approved by a regulatory body [65]. In the US there are only two categories of this type of product: Cellular and Gene Therapy Products (accessed on 16 September 2024) [66]. Currently, there are 38 licensed cellular and gene therapy products (accessed on 16 September 2024) [66]. Overall, ATMP constitute only a small part of the pharmaceutical market and are mostly developed in academic centers [67].

Not every new ATMP medicinal product will have a chance to appear on the market. Some products will not successfully pass the preclinical phase, others will stop at the early phases of clinical trials. Only a few, such as Alofisel, containing expanded ADSC, will complete the registration process. It illustrates the wider problem concerning the ATMPs' development. First of all, the requirements of the regulatory bodies are identical, i.e., equally demanding for the different stages of the product development. Therefore, validation of each production stage must be carried out in accordance with many strict regulations at a very early stage of ATMP development. In the case of the standard medicine, there are fewer regulations to be watched and the general methodology is, in principle, the same—typical for chemical substances. In the case of advanced therapy medicinal products (ATMPs), the validation plan is in most cases individual, which is associated with a significantly greater investment of time and costs and is always associated with a longer path to regulatory approval. It is also burdened with the risk of additional requirements. Furthermore, the EMA recommendations dedicated to ATMPs change dynamically. It should be mentioned that the "Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials" (EMA/CAT/123573/2024), currently required by the Clinical Trials Registration Offices, was announced on March 2024 as a draft with no date for coming into effect, while the previous version, from January 2019 (EMA/CAT/852602/2018), remains at the draft stage, also with no date for coming into effect. Those guidelines are accompanied by many other obligatory recommendations—frequently changing and not always entirely consistent with each other. Taking into account, that because of the species-determined active substance, the preclinical proof of concept based on animal observations is far from the real situation, as pharmaceutical companies are not interested in investing in ATIMP development during the early stages of clinical trials. Therefore, in this phase, the financial and organizational burden falls on the shoulders of the research centers, which is a serious obstacle to ATIMP being introduced to the market.

After entering the keyword “cell therapy” the [clinicaltrials.gov](https://clinicaltrials.gov) website searches for 8266 completed in phases spanning from early phase 1 to phase 4 (Figure 3). After introducing an additional filter, “diabetic foot” only 24 trials were found (as of 24 October 2024) (Table 1).



**Figure 3.** Number of MSC-related clinical trials found in [clinicaltrials.gov](https://clinicaltrials.gov) database with “cell therapy” keyword, and “completed” filter. Clinical trials were divided according to the declared trial phase into: early phase 1; phase 1/2; phase 2; phase 2/3; phase 3; and phase 4. †—“N/A” trial phase information was deemed “not applicable” in [clinicaltrials.gov](https://clinicaltrials.gov); ++—“N/D” no data were provided regarding the trial phase in [clinicaltrials.gov](https://clinicaltrials.gov) database.

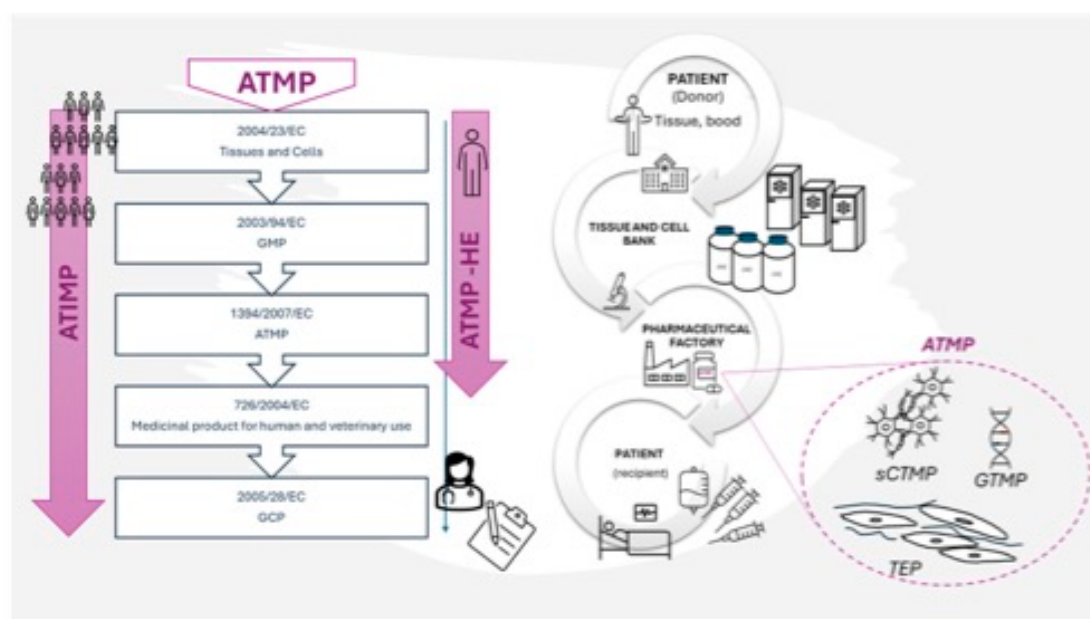
**Table 1.** Completed clinical trials listed in the [clinicaltrials.gov](https://clinicaltrials.gov) database, which uses cells in the therapy of diabetic foot syndrome.

| NCT Number  | MSC Type            | Phases          | Completion Date   |
|-------------|---------------------|-----------------|-------------------|
| NCT01065337 | BMSC                | PHASE2          | 1 February 2009   |
| NCT02619877 | ADSC                | PHASE2          | 1 October 2016    |
| NCT02070835 | other               | N/A †           | 1 December 2022   |
| NCT03183804 | ADSC                | N/D ++          | 24 October 2018   |
| NCT06373809 | PDSC                | EARLY_PHASE1    | 31 March 2024     |
| NCT02092870 | ADSC                | PHASE2          | 9 September 2019  |
| NCT02799121 | other               | PHASE4          | 29 March 2019     |
| NCT03881254 | other               | N/A †           | 28 July 2021      |
| NCT03276312 | ADSC/Adipose tissue | N/A †           | 1 March 2018      |
| NCT00536744 | other               | PHASE3          | 1 September 2010  |
| NCT04255004 | other               | N/A †           | 1 December 2019   |
| NCT04633642 | other               | N/A †           | 31 December 2019  |
| NCT03183726 | ADSC                | N/D ++          | 31 July 2017      |
| NCT03865394 | ADSC                | PHASE1   PHASE2 | 30 September 2021 |
| NCT01596920 | other               | PHASE4          | 1 March 2014      |
| NCT00872326 | BMSC                | PHASE1   PHASE2 | 1 May 2009        |
| NCT00987363 | BMSC                | PHASE1   PHASE2 | 1 March 2013      |
| NCT02224742 | other               | PHASE4          | 1 May 2018        |
| NCT02329366 | other               | N/D ++          | 1 December 2020   |
| NCT03547635 | other               | N/A †           | 26 February 2019  |
| NCT03636867 | other               | N/D ++          | 31 August 2020    |
| NCT03267784 | other MSC           | PHASE1   PHASE2 | 29 June 2020      |
| NCT01232673 | BMSC                | PHASE2          | 1 December 2010   |
| NCT01113307 | other               | N/D ++          | 1 September 2011  |

†—trial phase information was deemed “not applicable” in [clinicaltrials.gov](https://clinicaltrials.gov). ++—no data were provided regarding the trial phase in [clinicaltrials.gov](https://clinicaltrials.gov) database.



There are two legislative pathways regulating the application of ATMP to patients. The first describes the use of ATMP in a clinical study (also called Advanced Therapy Investigational Medicinal Product when undergoing clinical study—ATIMP). The second is the use of ATMP in a hospital-exemption (ATMP-HE) route where the ATMP is prepared on request, under the responsibility of the doctor, according to the individual needs of the patient (Figure 4).



**Figure 4.** An exemplary route of biological material for ATMP in connection with legal regulations. Only the most fundamental regulations are indicated.

The estimated time that may pass from the idea to launching the new product on the market lasts on average 15 years and it consumes large amount of financial resources [67,68].

It was expected that, due to their properties, cell-based products will enable the treatment of patients for whom current therapies cannot bring results or their effects are unsatisfactory. Regulatory authorities are aware of the unmet needs of the health market and are taking steps to accelerate the evaluation of new drugs. However, the legal requirements for ATMP consist of a plethora of documents and regulations, many of which are often intertwined. This leads to ambiguous interpretations hindering the development and approval processes.

Production of ATMP takes place in the pharmaceutical factory (PhF) that meets the requirements of the directive that regulates the good manufacturing practice (GMP) rules in respect of medicinal products for human use [69]. However, a pharmaceutical factory cannot accept biological material as a starting material for ATMP production directly from a recruiting center (clinic). Such a material has to go through the Tissue and Cell Bank (TCB) where it is qualified and accepted for further use (Figure 4). In Poland, entities that own ATMP factories are sometimes also registered as Tissue and Cell Banks and are therefore subjected to the regulations of European Commission: 2004/23/EC (directive on tissue and cells banking) [70], 2002/98/EC (directive on human blood banking) [71], or both, as well as the national regulatory system Biological material, e.g., adipose tissue, is verified twice, first in TCB and then in PhF. Then, after the positive qualification in PhF, the biological material (e.g., adipose tissue) is subjected to the cell isolation process (usually using the enzymatic method). After isolation, the final cell-containing product

can be prepared in a straight line, or cells are subjected to other manipulations. Under the regulations, even if nothing more than cell culture is carried out for the purpose of increasing a cell population to obtain a desired cell resource, it is considered “substantial manipulation”. Potential more invasive interference, like gene transduction, may require a different, separate infrastructure. In the case of MSCs intended to be used in wound healing, there are no genetic manipulations proposed so far. However, the stage of cell culture is obligatory for MSCs isolated from adipose tissue not only because of the desired cell expansion but also in order to obtain the pure MSC immunophenotype [72]. During the ATMP manufacturing process, there are many steps where samples are acquired to perform quality control tests (e.g., sterility, determining the level of endotoxins, excluding mycoplasma). For a more detailed description of the manufacturing process using ADSC and quality control steps see Szablowska-Gadomska et al., 2023 [73].

To sum up, there are several regulatory bodies involved in the process of making MSC-based products available for the treatment of diabetic wounds. On the one hand, it makes the road to the patient complicated, costly, and time consuming. On the other hand, it is intended to ensure the safety of the treatment. Importantly, we are observing the growing understanding of the specificity of cell-based products by regulatory bodies. Especially, agencies such as FDA or EMA are constantly working on making regulations more and more adequate. It seems that the evidently growing interest in cell-based therapies for various clinical situations will guarantee the expected progress—for the benefit of their use in diabetic foot ulcer as well.

## 5. Allogeneic Products’ Advantages and Challenges

The rising research focus toward allogeneic applications in therapies using mesenchymal stem/stromal cells isolated from adipose tissue seemed to be a natural consequence of the already known positive effects of using allogeneic mesenchymal stem/stromal cells isolated from bone marrow. In immunological terms, mesenchymal stem/stromal cells belong to a special group of cells because they do not express or have significantly reduced expression of MHC class I and II and co-stimulatory molecules (CD40, CD80/CD86) [74]. According to the [clinicaltrials.gov](https://clinicaltrials.gov) website (keyword allogeneic MSC), 169 clinical trials using MSC in an allogeneic system are being conducted worldwide (accessed on 16 September 2024). There are also products available on the medical market containing allogeneic mesenchymal cells isolated from adipose tissue, e.g., Alofisel. Currently, more studies where MSC-based drug products are subsequently administered in an allogeneic setting are conducted.

The path to development of the allogeneic MSC therapies is related to their described and extensively studied immunomodulatory properties [75]. ATMPs based on allogeneic material provide a chance to manufacture the product even when the recipient-patient’s health prevents the collection process, as may happen in the case of autologous products. Even a relatively minimally invasive procedure such as liposuction may pose a risk for patients with tendencies for the occurrence of chronic wounds (e.g., patients suffering from diabetes).

Allogeneic ATMPs also have a greater potential to be available in a shorter time, almost immediately. There is no need to wait for a long process of cell isolation and multiplication because they can be performed even before qualifying the recipient-patient for treatment.

The allogeneic system makes it possible to obtain cells from waste material, e.g., after liposuction, which would be of no use for donor-patients. Such material from healthy donors, after positive qualification, can be a valuable source of cells or a secretome for patients with health deficits.

However, despite the ever-growing research interest in MSC-based therapies, from a PhF’s point of view, the allogeneic ATMPs pose quite a challenge. In case of direct autologous transplantations (without isolation, manipulation, etc.), in Poland, the acceptance criteria are well-defined and classified by national regulatory system, whereas for ATMP production (especially allogeneic) the biological material has to undergo additional selec-



tion according to criteria based on Directive 2004/23/EC—Setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells [70].

Therefore, in addition to serological tests, physical examination and diagnostic tests are performed to exclude the most common cancer diseases, e.g., breast cancer, prostate cancer, uterus cancer, and lung cancer. The latter are also indicated by cancer marker tests. This approach toward potential donors of biological material may be seen as an advantage as it provides access to a wider panel of tests that are not routinely performed diagnostic tests but that have a significant preventive effect.

Regardless of whether therapies based on the administration of live cells or their secretome will ultimately prove to be the most effective therapies, the possibility of using an allogeneic system is critically important, especially in applications in diabetic patients.

## 6. Practical Issues Related to ATMP Release, and Its Delivery from the PhF to the Patient

A short shelf-life and strict requirements for storage and transport conditions of ATMP intended for immediate application after production pose a serious challenge for manufacturers and clinicians. The expiration date of such products varies and depends on the specificity of the cells themselves as well as the excipient in which the cells are suspended. Most often, the shelf-life spans a dozen to several dozen hours from the time the products are manufactured. This is especially important when the distance between the PhF and the clinic is long. A solution may be to transport deep-frozen products and defrost them in the clinic at the patient's bedside. However, this method requires the use of cryoprotectants (e.g., DMSO), which may sometimes pose an additional risk to patients. In addition, the condition of freshly thawed cells may be worse, compared to those obtained directly from the culture dish (for purely biological reasons) and may result in a weakened therapeutic effect. François et al. showed that the percentage of viability MSC collected from continuous culture was about 90%, while their freshly thawed counterparts dropped to about 40%. They also found a deterioration in the immunosuppressive properties of the tested cells [76]. Some authors even suggest that the poorer properties of the cells after thawing may be the reason for the unsatisfactory results of clinical trials with their participation [77–79]. Therefore, it is recommended to consider this important aspect during the planning of the manufacturing process, creation of the new product specification, and later during the testing of cell parameters at the stage of application of the finished product. This will allow us to avoid the high costs of clinical trials due to suboptimal use of the potential of MSC cells during manufacturing and the risk of interference resulting from the impact of the thawing process itself.

The use of a cell secretome may be a cheaper and logistically easier solution due to feasible normalization of the storage conditions [80]. MSCs can secrete substances capable of, among others: regulating the functions of immune system cells, and improving the healing microenvironment [81]. Medicinal products containing cell secretome may be perceived as safer from a microbiological point of view versus cells because they could be sterilized (e.g., by filtration). Additionally, it would be easier to standardize the secretome in terms of the tested parameters, e.g., dose and potency. However, the use of cells has the advantage that their secretome may vary depending on changing environmental conditions [82]. Therefore, a specific cell response to the local environment of the wound might be expected and result in secreting a specific and the most required cocktail of factors.

In the case of medicinal products with live cells, it is permissible and is often the practice to administer them to the patient before receiving all the results of analytical tests, including the initial certification and release for administration. Then, the final certification is carried out by a Qualified Person (QP) after acquiring all test results. This is due to the required sterility tests, which according to pharmacopoeial methods require 14 days of incubation, while the shelf life of the product may be several hours. More information on this subject can be found in the work of Szablowska-Gadomska et al. [73].

The biological properties of MSCs are difficult to assess in animal models, especially when considering the immunomodulatory properties as their main therapeutic value [81,83]. Using the immunocompromised animals required for human MSC testing, to assess the dosage of MSCs while preparing the ATMP, e.g., for DFU treatment, may result in a loss of information regarding the immunomodulatory properties of MSCs and its relation to the dosage. Thus, ATMPs containing live MSCs face a critical issue of dosage selection. In [clinicaltrials.gov](https://clinicaltrials.gov), the variety of doses used in registered clinical studies ranges from 2 to  $100 \times 10^6$ /mL applied both systemically or locally per injection or directly on wounds (accessed on 19 February 2024).

One of the concerns expressed by regulatory bodies is the supposed cancerogenic potential of MSC. Therefore, EMA guidelines were issued in 2024 regarding the requirements for preclinical ATMP studies involving an extensive tumorigenic and immunogenic safety assessment (EMA/CAT/123753/2024). However, MSCs show almost negligible immunogenicity and by some are even called immune-evasive [75,84]. Moreover, unmodified MSCs, that are often used in cell therapies, which underwent only minimal in vitro manipulations such as isolation and expansion retain their genetic stability. The reported rare tumorigenic events were not caused by MSC but by the ATIMP contamination with tumor cells [85]. Therefore, legal bodies have applied an especially high pressure on researchers to ascertain the identity of cells used in ATIMP as the origin and purity of starting cellular material is one of the key factors in the safety of the ATIMP.

## 7. The Ways of Administration of MSC to Intended Locations

The utilization of MSC as a therapeutic modality for chronic and recalcitrant wounds in diabetic patients is an active area of investigation aimed at enhancing its therapeutic potential and expediting tissue regeneration. The method of delivering MSC to the intended location of action is a key topic of scientific interest. Through a comprehensive review of existing literature and clinical studies we can distinguish two main ways of administering MSC, which are a systemic delivery and a local administration.

Local administration includes the direct injection of MSC into the wound alone or the inclusion in a collagen sponge, on hydrogel scaffolds, or on other specially designed platforms.

Systemic delivery is divided into intravenous and arterial administration [45].

By investigating the complex differences between the mentioned two methods, we intend to analyze each modality's safety record, therapeutic utility, and effectiveness in relation to find the proper administration method for chronic wound therapy.

The utilization of specialized scaffolds as a platform for the local delivery of the stem cells has emerged as a promising therapeutic approach that has garnered significant attention in the scientific community and clinical trials. Regardless of the constituent materials utilized, the shared objective of these scaffolds is to promote tissue repair, improve stem cell survival, augment cell distribution within the wound and support their secretory functions [86]. The most advantageous modalities appropriate for the chosen biomaterials have been carefully determined and selected by the authors as part of the scope of this review.

Hydrogel scaffolds have emerged as a promising approach in regenerative medicine due to their biodegradability, ability to promote cellular survival, and controlled release of drugs and biomolecules [86,87]. Furthermore, hydrogel can be developed into a three-dimensional printing nano-architecture to create a sustainable and attractive stem cell niche, ensuring proper micro-structure and support. The three-dimensional extracellular matrix created by the Pluronic F-127 hydrogel exhibits excellent cellular affinity, making it a supportive environment for stem cell proliferation and it has been frequently utilized as a graft medium in cellular therapies with a high rate of success [88]. Collagen fibrils in hydrogels, which contain Arg-Gly-Asp (RGD) sequences, provide binding sites for cell interaction and adhesion [89]. Following this, MSC bound to the scaffolds can be minimally invasively injected at the site of a lesion, reducing the need for surgical intervention. Moreover, hydrogel scaffolds are capable of loading hydrophilic drugs and biomolecules, and fibrin hydrogel has been proposed as a means of improving the retention and stability



of BMSCs-exos in vivo [90]. However, one of the drawbacks associated with hydrogel scaffolds is that the release of drugs can be problematic, as uncontrolled diffusion, or unfavorable conditions, can lead to inadequate kinetics and delivery. In particular, molecules with low steric hindrance may diffuse uncontrollably, making them difficult to manage with precision. Additionally, the loading of hydrophobic drugs with limited affinity for the aqueous environment presents a significant limitation. However, it is possible to address these issues by incorporating bonds between drug molecules to regulate the rate of release. Depending on the strength of these bonds, they can be broken more or less easily, resulting in a more controlled delivery of stem cell biofactors.

In a study conducted by Shi et al., a chitosan/silk hydrogel was used in an in vivo animal setting. The resulting scaffold demonstrated desirable properties, including appropriate moisture retention and swelling characteristics. Studies have demonstrated the potential of GMSC-derived exosomes transferred in the environment of a chitosan/silk hydrogel in promoting wound healing in diabetic patients. The addition of GMSC-derived exosomes resulted in a significant reduction in wound size compared to both the control group and the group treated with hydrogel alone, indicating the potential of exosomes as a therapeutic agent for diabetic wound healing. Furthermore, it was observed that the group treated with hydrogel alone exhibited a significantly smaller wound size at both 1 and 2 weeks of treatment compared to the control group, highlighting the potential of hydrogel as a treatment option for wound healing [91].

Overall, hydrogel scaffolds offer a promising avenue for regenerative medicine, with further research needed to address the limitations and optimize their potential.

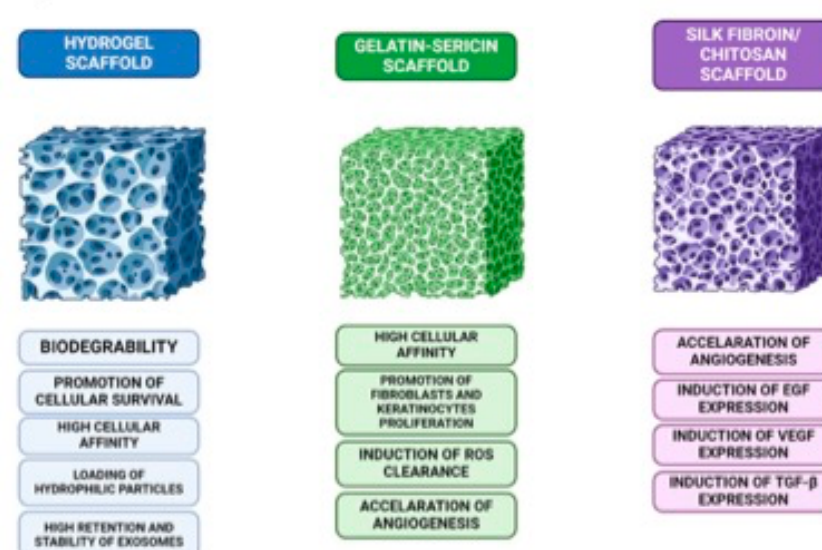
A widely used strategy in scaffold design is to utilize materials that can stimulate MSC and boost their secretory functions by modulating their molecular behavior. This category of materials is quite extensive, and for the purpose of this study, we selected the most promising candidates. Among these candidates is the gelatin-sericin (GS) scaffold, which is coated with laminin (GSL) and a silk fibroin/chitosan composite scaffold.

The combination of gelatin and sericin is capable of binding to cells, promoting cell proliferation in fibroblasts and keratinocytes. In vitro studies demonstrated that GS scaffolds had significantly increased cell proliferation when compared to non-scaffold models. Sericin has been found to enhance the migratory properties and adhesiveness of mammalian cells by activating the c-Jun pathway. It has also been suggested that it can increase cellular proliferation via a mechanism that has not yet been fully characterized, showing mitogenic properties. The GS scaffold promotes the clearance of cytotoxic reactive oxygen species, which can inhibit angiogenesis, and confers protection against oxidative stress to stem cells. Meanwhile, laminin, a component of the endothelial basal lamina, accelerates neovascularization in the wound bed, thereby enhancing wound healing and treatment efficacy. In the study conducted by Tyeb and colleagues, the authors demonstrated on a rat model that the MSC therapy group with GSL scaffold exhibited the most favorable outcomes in terms of wound healing, highlighting the potential of this approach as a therapeutic strategy for treating wounds in diabetic patients [92].

A silk fibroin/chitosan composite scaffold was used by Wu et al. to transfer adipose-derived stem/stromal cells (ADSC) to the wound site in diabetic rats. In this trial, there were a total of two control groups: one received only ASC grafts applied to the lesion, while the other received no grafts at all. Epidermal growth factor (EGF), tumor growth factor beta (TGF- $\beta$ ), and vascular endothelial growth factor (VEGF) were among the substances that the study group secreted in a higher amount than the control. The graft-only group's VEGF expression was considerably higher. These results provide further evidence supporting the use of such scaffolds for the administration of ADSCs (Figure 5) [93].

There have also been several attempts to deliver MSC to the site of a wound, with the addition of pharmacological molecules as stimulating factors. One of the more promising studies was conducted by Seo et al., who superficially applied Exendin-4 (Ex-4), a glucagon-like peptide-1 receptor agonist, to the wound area after injecting MSCs. Ex-4 is known to have beneficial effects on diabetes. In vitro angiogenesis assays demonstrated

that treatment with Ex-4 and ADSC-conditioned media (CM) improved migration, invasion, and proliferation of human endothelial cells. Combining a cell transplant with a topical application of a diabetes drug did not result in a decrease in blood glucose. Nevertheless, concomitant administration of Exendin-4 (Ex-4) and ADSC-conditioned media (CM) resulted in a significantly superior therapeutic effect compared to the individual treatments alone. Proliferation assays revealed a marked increase in proliferation following treatment with Ex-4, ADSC-CM, or the combination of both. The investigation demonstrated an increase in vascular endothelial growth factor (VEGF) expression in the wound area following treatment with Exendin-4 (Ex-4), ADSCs, or their combination. Nonetheless, co-administration of Ex-4 and ADSCs did not elicit a further enhancement in VEGF expression [94].



**Figure 5.** A comparison of three different scaffolds used in tissue engineering: hydrogel scaffolds, known for their biodegradability, cellular survival promotion, and exosome stability; gelatin-sericin scaffolds, which offer high cellular affinity, induce reactive oxygen species (ROS) clearance, and enhance fibroblast and keratinocyte proliferation; and silk fibroin/chitosan scaffolds, which accelerate angiogenesis and stimulate the expression of growth factors like EGF, VEGF, and TGF- $\beta$ , crucial for tissue regeneration.

The systemic administration of MSC remains poorly understood, with limited clinical trials exploring this route for treating diabetic foot ulcers. Unlike in systemic diseases like, for example, scleroderma, where MSCs are widely distributed and have been shown to halt internal fibrosis [95,96].

One such study, conducted by Yan et al., sought to directly compare intravenous (IV) administration with topical administration of MSCs in a rat model. Their findings shed light on the comparative efficacy and safety of these two modalities. In the research, wounds treated with bone marrow-derived MSC showcased faster healing rates in both the topical and intravenous (IV) administration groups compared to the control group throughout the study period ( $p < 0.05$ ). Notably, the IV group exhibited a significantly superior healing rate compared to the topical group at days 3 ( $p < 0.01$ ) and 10 ( $p < 0.05$ ). Additionally, histological examination revealed complete epithelialization in both the topical and IV groups by day 14, indicating the effectiveness of both administration methods in facilitating wound closure. These studies also have detected systemically administered cells in various organs such as the lungs, pancreas, liver, kidneys, and the wound site. Moreover, the study unveiled supplementary advantages linked with systemic transplantation of BM-MSCs, such as the



amelioration of hyperglycemia. These findings highlight the potential systemic effects of MSC therapy beyond wound healing, further emphasizing the importance of considering both local and systemic implications when choosing the administration route [97].

## 8. Summary

The utilization of MSC represents a propitious therapeutic avenue for wound healing. Nevertheless, further investigation is warranted to refine the technique of administration and to discern the optimal milieu for cellular delivery. Although the outcome of current research is encouraging, they are not suitable enough to determine whether this treatment modality is effective. Recent clinical trials investigating the use of MSCs in chronic wounds, such as DFU, have explored both autologous and allogeneic MSCs. Autologous MSCs, derived from the patient's own tissue, have demonstrated promising results in accelerating wound healing and reducing ulcer size. Studies, such as Tanios et al., reported significantly faster healing rates with autologous adipose-derived MSCs compared to standard care [98]. Meanwhile, trials with allogeneic MSCs, where cells are donated from another individual, have shown similar positive outcomes. For example, Uzun et al. reported the safe and effective use of allogeneic MSCs, with no significant immune reactions observed [99]. Both types of MSC therapies, autologous and allogeneic, exhibited enhanced healing capabilities in chronic wounds, though more research is needed to address long-term safety and efficacy. Therefore, future research ought to concentrate on improving MSC delivery to the wound site and investigating the underlying mechanisms by which we can maximize MSC's regenerative potential. It is believed that via continued research and development efforts, MSC-based therapies may be more widely adopted in the clinical setting, improving the clinical outcomes of patients with chronic wounds.

**Author Contributions:** Conceptualization, D.S. and B.M.-R.; methodology, D.S. and B.M.-R.; validation, D.S., B.M.-R., S.R., I.S.-G. and M.L.-S.; formal analysis, D.S.; investigation, D.S. and B.M.-R.; data curation, D.S., I.S.-G., M.L.-S. and B.M.-R.; writing—original draft preparation, D.S., I.S.-G., A.N.-S. and M.G.; writing—review and editing, D.S., I.S.-G., A.N.-S., M.G., S.R., M.L.-S., L.C. and B.M.-R.; visualization, D.S., P.Z., I.S.-G., S.R. and B.M.-R.; supervision, M.L.-S., L.C., W.S.Z. and B.M.-R.; project administration, D.S., I.S.-G. and B.M.-R. All authors have read and agreed to the published version of the manuscript.

**Funding:** The research was funded by the Medical Research Agency, Poland from state budget funds, grant number 2021/ABM/03/00037.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## References

1. Sun, H.; Saeedi, P.; Karuranga, S.; Pinkepank, M.; Ogurtsova, K.; Duncan, B.B.; Stein, C.; Basit, A.; Chan, J.C.N.; Mbanya, J.C.; et al. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes Res. Clin. Pract.* **2022**, *183*, 109119. [\[CrossRef\]](#) [\[PubMed\]](#)
2. Wu, Y.; Chen, L.; Scott, P.G.; Tredget, E.E. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* **2007**, *25*, 2648–2659. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Yang, H.Y.; Fierro, F.; So, M.; Yoon, D.J.; Nguyen, A.V.; Gallegos, A.; Bagoos, M.D.; Rojo-Castro, T.; Alex, A.; Stewart, H.; et al. Combination product of dermal matrix, human mesenchymal stem cells, and timolol promotes diabetic wound healing in mice. *Stem Cells Transl. Med.* **2020**, *9*, 1353–1364. [\[CrossRef\]](#) [\[PubMed\]](#)
4. Liu, J.; Gao, J.; Liang, Z.; Gao, C.; Niu, Q.; Wu, F.; Zhang, L. Mesenchymal stem cells and their microenvironment. *Stem Cell Res. Ther.* **2022**, *13*, 429. [\[CrossRef\]](#)
5. Jo, H.; Brito, S.; Kwak, B.M.; Park, S.; Lee, M.G.; Bin, B.H. Applications of Mesenchymal Stem Cells in Skin Regeneration and Rejuvenation. *Int. J. Mol. Sci.* **2021**, *22*, 2410. [\[CrossRef\]](#)
6. Wu, X.; Jiang, J.; Gu, Z.; Zhang, J.; Chen, Y.; Liu, X. Mesenchymal stromal cell therapies: Immunomodulatory properties and clinical progress. *Stem Cell Res. Ther.* **2020**, *11*, 345. [\[CrossRef\]](#)
7. Wilson, A.J.; Rand, E.; Webster, A.J.; Genever, P.G. Characterisation of mesenchymal stromal cells in clinical trial reports: Analysis of published descriptors. *Stem Cell Res. Ther.* **2021**, *12*, 360. [\[CrossRef\]](#)
8. Zhang, S.; Chen, L.; Zhang, G.; Zhang, B. Umbilical cord-matrix stem cells induce the functional restoration of vascular endothelial cells and enhance skin wound healing in diabetic mice via the polarized macrophages. *Stem Cell Res. Ther.* **2020**, *11*, 39. [\[CrossRef\]](#)

9. Jodheea-Jutton, A.; Hindocha, S.; Bhaw-Luximon, A. Health economics of diabetic foot ulcer and recent trends to accelerate treatment. *Foot* **2022**, *52*, 101909. [\[CrossRef\]](#)
10. Ho, J.; Yue, D.; Cheema, U.; Hsia, H.C.; Dardik, A. Innovations in Stem Cell Therapy for Diabetic Wound Healing. *Adv. Wound Care* **2023**, *12*, 626–643. [\[CrossRef\]](#)
11. Asko Andersen, J.; Rasmussen, A.; Frimodt-Møller, M.; Engberg, S.; Steeneveld, E.; Kirketerp-Møller, K.; O'Brien, T.; Rossing, P. Novel topical allogeneic bone-marrow-derived mesenchymal stem cell treatment of hard-to-heal diabetic foot ulcers: A proof of concept study. *Stem Cell Res. Ther.* **2022**, *13*, 280. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Chiang, K.J.; Chiu, L.C.; Kang, Y.N.; Chen, C. Autologous Stem Cell Therapy for Chronic Lower Extremity Wounds: A Meta-Analysis of Randomized Controlled Trials. *Cells* **2021**, *10*, 3307. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Song, Y.; You, Y.; Xu, X.; Lu, J.; Huang, X.; Zhang, J.; Zhu, L.; Hu, J.; Wu, X.; Xu, X.; et al. Adipose-Derived Mesenchymal Stem Cell-Derived Exosomes Biopotentiates Extracellular Matrix Hydrogels Accelerate Diabetic Wound Healing and Skin Regeneration. *Adv. Sci.* **2023**, *10*, e2304023. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Verdi, J.; Shirian, S.; Saleh, M.; Khadem Haghighian, H.; Kavianpour, M. Mesenchymal Stem Cells Regenerate Diabetic Foot Ulcers: A Review Article. *World J. Plast. Surg.* **2022**, *11*, 12–22. [\[CrossRef\]](#)
15. Rai, V.; Moellmer, R.; Agrawal, D.K. Stem Cells and Angiogenesis: Implications and Limitations in Enhancing Chronic Diabetic Foot Ulcer Healing. *Cells* **2022**, *11*, 2287. [\[CrossRef\]](#)
16. Li, H.; Zhu, H.; Ge, T.; Wang, Z.; Zhang, C. Mesenchymal Stem Cell-Based Therapy for Diabetes Mellitus: Enhancement Strategies and Future Perspectives. *Stem Cell Rev. Rep.* **2021**, *17*, 1552–1569. [\[CrossRef\]](#)
17. Jiang, M.; Jiang, X.; Li, H.; Zhang, C.; Zhang, Z.; Wu, C.; Zhang, J.; Hu, J.; Zhang, J. The role of mesenchymal stem cell-derived EVs in diabetic wound healing. *Front. Immunol.* **2023**, *14*, 1136098. [\[CrossRef\]](#)
18. Jeffcoate, W.J.; Vileikyte, L.; Boyko, E.J.; Armstrong, D.G.; Boulton, A.J.M. Current Challenges and Opportunities in the Prevention and Management of Diabetic Foot Ulcers. *Diabetes Care* **2018**, *41*, 645–652. [\[CrossRef\]](#)
19. Nalisa, D.L.; Moneruzzaman, M.; Changwe, G.J.; Mobet, Y.; Li, L.P.; Ma, Y.J.; Jiang, H.W. Stem Cell Therapy for Diabetic Foot Ulcers: Theory and Practice. *J. Diabetes Res.* **2022**, *2022*, 6028743. [\[CrossRef\]](#)
20. Shi, Y.; Vanhoutte, P.M. Macro- and microvascular endothelial dysfunction in diabetes. *J. Diabetes* **2017**, *9*, 434–449. [\[CrossRef\]](#)
21. Khalid, M.; Petroianu, G.; Adem, A. Advanced Glycation End Products and Diabetes Mellitus: Mechanisms and Perspectives. *Biomolecules* **2022**, *12*, 542. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Nowotny, K.; Jung, T.; Hohn, A.; Weber, D.; Grune, T. Advanced glycation end products and oxidative stress in type 2 diabetes mellitus. *Biomolecules* **2015**, *5*, 194–222. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Yamagishi, S.; Maeda, S.; Matsui, T.; Ueda, S.; Fukami, K.; Okuda, S. Role of advanced glycation end products (AGEs) and oxidative stress in vascular complications in diabetes. *Biochim. Biophys. Acta* **2012**, *1820*, 663–671. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Knapp, M.; Tu, X.; Wu, R. Vascular endothelial dysfunction, a major mediator in diabetic cardiomyopathy. *Acta Pharmacol. Sin.* **2019**, *40*, 1–8. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Sawaya, A.P.; Stone, R.C.; Brooks, S.R.; Pastar, I.; Jozic, I.; Hasneen, K.; O'Neill, K.; Mehdizadeh, S.; Head, C.R.; Strbo, N.; et al. Deregulated immune cell recruitment orchestrated by FOXM1 impairs human diabetic wound healing. *Nat. Commun.* **2020**, *11*, 4678. [\[CrossRef\]](#)
26. Mrozikiewicz-Rakowska, B.; Szablowska-Gadomska, I.; Cysewski, D.; Rudzinski, S.; Ploski, R.; Gasperowicz, P.; Konarzewska, M.; Zielinski, J.; Mieczkowski, M.; Sienko, D.; et al. Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact. *Int. J. Mol. Sci.* **2023**, *24*, 1472. [\[CrossRef\]](#)
27. Sloan, G.; Selvarajah, D.; Tesfaye, S. Pathogenesis, diagnosis and clinical management of diabetic sensorimotor peripheral neuropathy. *Nat. Rev. Endocrinol.* **2021**, *17*, 400–420. [\[CrossRef\]](#)
28. Lin, Q.; Li, K.; Chen, Y.; Xie, J.; Wu, C.; Cui, C.; Deng, B. Oxidative Stress in Diabetic Peripheral Neuropathy: Pathway and Mechanism-Based Treatment. *Mol. Neurobiol.* **2023**, *60*, 4574–4594. [\[CrossRef\]](#)
29. Watroba, M.; Grabowska, A.D.; Szukiewicz, D. Effects of Diabetes Mellitus-Related Dysglycemia on the Functions of Blood-Brain Barrier and the Risk of Dementia. *Int. J. Mol. Sci.* **2023**, *24*, 10069. [\[CrossRef\]](#)
30. Maruhashi, T.; Higashi, Y. Pathophysiological Association between Diabetes Mellitus and Endothelial Dysfunction. *Antioxidants* **2021**, *10*, 1306. [\[CrossRef\]](#)
31. Kaur, M.; Misra, S.; Swarnkar, P.; Patel, P.; Das Kurmi, B.; Das Gupta, G.; Singh, A. Understanding the role of hyperglycemia and the molecular mechanism associated with diabetic neuropathy and possible therapeutic strategies. *Biochem. Pharmacol.* **2023**, *215*, 115723. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Caturano, A.; D'Angelo, M.; Mormone, A.; Russo, V.; Mollica, M.P.; Salvatore, T.; Galiero, R.; Rinaldi, L.; Vetrano, E.; Marfella, R.; et al. Oxidative Stress in Type 2 Diabetes: Impacts from Pathogenesis to Lifestyle Modifications. *Curr. Issues Mol. Biol.* **2023**, *45*, 6651–6666. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Das, A.K.; Kalra, S.; Punyani, H.; Deshmukh, S.; Taur, S. 'Oxidative stress'-A new target in the management of diabetes mellitus. *J. Family Med. Prim. Care* **2023**, *12*, 2552–2557. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Monteiro-Soares, M.; Boyko, E.J.; Ribeiro, J.; Ribeiro, I.; Dinis-Ribeiro, M. Predictive factors for diabetic foot ulceration: A systematic review. *Diabetes Metab. Res. Rev.* **2012**, *28*, 574–600. [\[CrossRef\]](#)
35. Van Dam, P.S.; Cotter, M.A.; Bravenboer, B.; Cameron, N.E. Pathogenesis of diabetic neuropathy: Focus on neurovascular mechanisms. *Eur. J. Pharmacol.* **2013**, *719*, 180–186. [\[CrossRef\]](#)



36. Ahmed, L.A.; Al-Massri, K.F. Exploring the Role of Mesenchymal Stem Cell-Derived Exosomes in Diabetic and Chemotherapy-Induced Peripheral Neuropathy. *Mol. Neurobiol.* **2024**, *61*, 5916–5927. [\[CrossRef\]](#)
37. Reddy, V.P. Oxidative Stress in Health and Disease. *Biomedicines* **2023**, *11*, 2925. [\[CrossRef\]](#)
38. Riaz, B.; Sohn, S. Neutrophils in Inflammatory Diseases: Unraveling the Impact of Their Derived Molecules and Heterogeneity. *Cells* **2023**, *12*, 2621. [\[CrossRef\]](#)
39. Tsioumpekou, M.; Krijgsman, D.; Leusen, J.H.W.; Olofson, P.A. The Role of Cytokines in Neutrophil Development, Tissue Homing, Function and Plasticity in Health and Disease. *Cells* **2023**, *12*, 1981. [\[CrossRef\]](#)
40. Carnevale, S.; Di Ceglie, I.; Grieco, G.; Rigatelli, A.; Bonavita, E.; Jaillon, S. Neutrophil diversity in inflammation and cancer. *Front. Immunol.* **2023**, *14*, 1180810. [\[CrossRef\]](#)
41. Zhou, J.; Chen, B. Retinal Cell Damage in Diabetic Retinopathy. *Cells* **2023**, *12*, 1342. [\[CrossRef\]](#) [\[PubMed\]](#)
42. Li, H.; Liu, C.; Zhang, J.; Wang, W.; Cheng, W.; Yang, R.; Huang, A.M.; Liang, J.; Guo, J.; Liu, Z. The association of homocysteine level with the risk of diabetic nephropathy and diabetic retinopathy in NHANES. *Acta Diabetol.* **2023**, *60*, 907–916. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Goldney, J.; Sargeant, J.A.; Davies, M.J. Incretins and microvascular complications of diabetes: Neuropathy, nephropathy, retinopathy and microangiopathy. *Diabetologia* **2023**, *66*, 1832–1845. [\[CrossRef\]](#)
44. Raja, J.M.; Maturana, M.A.; Kayali, S.; Khouzam, A.; Efeovbokhan, N. Diabetic foot ulcer: A comprehensive review of pathophysiology and management modalities. *World J. Clin. Cases* **2023**, *11*, 1684–1693. [\[CrossRef\]](#)
45. Yu, X.; Liu, P.; Li, Z.; Zhang, Z. Function and mechanism of mesenchymal stem cells in the healing of diabetic foot wounds. *Front. Endocrinol.* **2023**, *14*, 1099310. [\[CrossRef\]](#)
46. Lopes, L.; Setia, O.; Aurshina, A.; Liu, S.; Hu, H.; Isaji, T.; Liu, H.; Wang, T.; Ono, S.; Guo, X.; et al. Stem cell therapy for diabetic foot ulcers: A review of preclinical and clinical research. *Stem Cell Res. Ther.* **2018**, *9*, 188. [\[CrossRef\]](#)
47. Jin, Q.H.; Kim, H.K.; Na, J.Y.; Jin, C.; Seon, J.K. Anti-inflammatory effects of mesenchymal stem cell-conditioned media inhibited macrophages activation in vitro. *Sci. Rep.* **2022**, *12*, 4754. [\[CrossRef\]](#)
48. Yang, S.H.; Park, M.J.; Yoon, I.H.; Kim, S.Y.; Hong, S.H.; Shin, J.Y.; Nam, H.Y.; Kim, Y.H.; Kim, B.; Park, C.G. Soluble mediators from mesenchymal stem cells suppress T cell proliferation by inducing IL-10. *Exp. Mol. Med.* **2009**, *41*, 315–324. [\[CrossRef\]](#)
49. Nasef, A.; Chapel, A.; Mazurier, C.; Bouchet, S.; Lopez, M.; Mathieu, N.; Sensebe, L.; Zhang, Y.; Gorin, N.C.; Thierry, D.; et al. Identification of IL-10 and TGF- $\beta$  transcripts involved in the inhibition of T-lymphocyte proliferation during cell contact with human mesenchymal stem cells. *Gene Expr.* **2007**, *13*, 217–226. [\[CrossRef\]](#)
50. Muinos-Lopez, E.; Ripalda-Cemborain, P.; Lopez-Martinez, T.; Gonzalez-Gil, A.B.; Lamo-Espinosa, J.M.; Valenti, A.; Mortlock, D.P.; Valenti, J.R.; Prosper, F.; Granero-Molto, F. Hypoxia and Reactive Oxygen Species Homeostasis in Mesenchymal Progenitor Cells Define a Molecular Mechanism for Fracture Nonunion. *Stem Cells* **2016**, *34*, 2342–2353. [\[CrossRef\]](#)
51. Pajarinen, J.; Lin, T.; Gibon, E.; Kohno, Y.; Maruyama, M.; Nathan, K.; Lu, L.; Yao, Z.; Goodman, S.B. Mesenchymal stem cell-macrophage crosstalk and bone healing. *Biomaterials* **2019**, *196*, 80–89. [\[CrossRef\]](#) [\[PubMed\]](#)
52. Cho, D.I.; Kim, M.R.; Jeong, H.Y.; Jeong, H.C.; Jeong, M.H.; Yoon, S.H.; Kim, Y.S.; Ahn, Y. Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. *Exp. Mol. Med.* **2014**, *46*, e70. [\[CrossRef\]](#) [\[PubMed\]](#)
53. Saheli, M.; Bayat, M.; Ganji, R.; Hendudari, F.; Kheirjou, R.; Pakzad, M.; Najari, B.; Piryaee, A. Human mesenchymal stem cells-conditioned medium improves diabetic wound healing mainly through modulating fibroblast behaviors. *Arch. Dermatol. Res.* **2020**, *312*, 325–336. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Yates, C.C.; Rodrigues, M.; Nuschke, A.; Johnson, Z.I.; Whaley, D.; Stolz, D.; Newsome, J.; Wells, A. Multipotent stromal cells/mesenchymal stem cells and fibroblasts combine to minimize skin hypertrophic scarring. *Stem Cell Res. Ther.* **2017**, *8*, 193. [\[CrossRef\]](#) [\[PubMed\]](#)
55. Li, Y.; Zhang, J.; Shi, J.; Liu, K.; Wang, X.; Jia, Y.; He, T.; Shen, K.; Wang, Y.; Liu, J.; et al. Exosomes derived from human adipose mesenchymal stem cells attenuate hypertrophic scar fibrosis by miR-192-5p/IL-17RA/Smad axis. *Stem Cell Res. Ther.* **2021**, *12*, 221. [\[CrossRef\]](#)
56. Johnson, K.E.; Wilgus, T.A. Vascular Endothelial Growth Factor and Angiogenesis in the Regulation of Cutaneous Wound Repair. *Adv. Wound Care* **2014**, *3*, 647–661. [\[CrossRef\]](#)
57. Tan, Q.; Chen, B.; Yan, X.; Lin, Y.; Xiao, Z.; Hou, X.; Dai, J. Promotion of diabetic wound healing by collagen scaffold with collagen-binding vascular endothelial growth factor in a diabetic rat model. *J. Tissue Eng. Regen. Med.* **2014**, *8*, 195–201. [\[CrossRef\]](#)
58. Gonzalez, A.C.; Costa, T.F.; Andrade, Z.A.; Medrado, A.R. Wound healing—A literature review. *An. Bras. Dermatol.* **2016**, *91*, 614–620. [\[CrossRef\]](#)
59. Zhou, P.; Yang, C.; Zhang, S.; Ke, Z.X.; Chen, D.X.; Li, Y.Q.; Li, Q. The Imbalance of MMP-2/TIMP-2 and MMP-9/TIMP-1 Contributes to Collagen Deposition Disorder in Diabetic Non-Injured Skin. *Front. Endocrinol.* **2021**, *12*, 734485. [\[CrossRef\]](#)
60. Lozito, T.P.; Jackson, W.M.; Nesti, L.J.; Tuan, R.S. Human mesenchymal stem cells generate a distinct pericellular zone of MMP activities via binding of MMPs and secretion of high levels of TIMPs. *Matrix Biol.* **2014**, *34*, 132–143. [\[CrossRef\]](#)
61. Li, M.; Zhao, Y.; Hao, H.; Dai, H.; Han, Q.; Tong, C.; Liu, J.; Han, W.; Fu, X. Mesenchymal stem cell-conditioned medium improves the proliferation and migration of keratinocytes in a diabetes-like microenvironment. *Int. J. Low. Extrem. Wounds* **2015**, *14*, 73–86. [\[CrossRef\]](#) [\[PubMed\]](#)

62. European Commission. Regulation (EC) No 1394/2007 of the European Parliament and of the Council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004. *Off. J. Eur. Union* **2007**, *50*, 121–137.
63. European Commission. Regulation (EC) No 726/2004 of the European Parliament and of the Council of 31 March 2004 laying down Union procedures for the authorisation and supervision of medicinal products for human use and establishing a European Medicines Agency. *Off. J. Eur. Union* **2004**, *47*, 1–33.
64. European Commission. Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use. *Off. J. Eur. Union* **2001**, *311*, 67–128.
65. Bellino, S.; La Salvia, A.; Cometa, M.F.; Botta, R. Cell-based medicinal products approved in the European Union: Current evidence and perspectives. *Front. Pharmacol.* **2023**, *14*, 1200808. [\[CrossRef\]](#)
66. FDA. Approved Cellular and Gene Therapy Products. Available online: <https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products> (accessed on 16 September 2024).
67. Elsanhoury, A.; Sanzenbacher, R.; Reinke, P.; Abou-El-Enein, M. Accelerating Patients' Access to Advanced Therapies in the EU. *Mol. Ther.-Methods Clin. Dev.* **2017**, *7*, 15–19. [\[CrossRef\]](#)
68. Warreth, S.; Harris, E. The regulatory landscape for ATMPs in the EU and US: A comparison. *Level 3* **2020**, *15*, 5.
69. European Commission. Commission Directive 2003/94/EC of 8 October 2003 laying down the principles and guidelines of good manufacturing practice in respect of medicinal products for human use and investigational medicinal products for human use. *Off. J. Eur. Union* **2003**, *262*, 22–26.
70. European Commission. Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells. *Off. J. Eur. Union* **2004**, *47*, 48–58.
71. European Commission. Directive 2002/98/EC of the European Parliament and of the Council of 27 January 2003 setting standards of quality and safety for the collection, testing, processing, storage and distribution of human blood and blood components and amending Directive 2001/83/EC. *Off. J. Eur. Union* **2003**, *33*, 30–40.
72. Zuk, P. Adipose-Derived Stem Cells in Tissue Regeneration: A Review. *ISRN Stem Cells* **2013**, *2013*, 713959. [\[CrossRef\]](#)
73. Szablowska-Gadomska, I.; Humiecka, M.; Brzezicka, J.; Chrościcka, A.; Placzowska, J.; Oldak, T.; Lewandowska-Szumiel, M. Microbiological Aspects of Pharmaceutical Manufacturing of Adipose-Derived Stem Cell-Based Medicinal Products. *Cells* **2023**, *12*, 680. [\[CrossRef\]](#) [\[PubMed\]](#)
74. Wang, Y.; Huang, J.; Gong, L.; Yu, D.; An, C.; Bunpetch, V.; Dai, J.; Huang, H.; Zou, X.; Ouyang, H.; et al. The Plasticity of Mesenchymal Stem Cells in Regulating Surface HLA-I. *iScience* **2019**, *15*, 66–78. [\[CrossRef\]](#) [\[PubMed\]](#)
75. Ankrum, J.A.; Ong, J.F.; Karp, J.M. Mesenchymal stem cells: Immune evasive, not immune privileged. *Nat. Biotechnol.* **2014**, *32*, 252–260. [\[CrossRef\]](#)
76. Francois, M.; Copland, I.B.; Yuan, S.; Romieu-Mourez, R.; Waller, E.K.; Galipeau, J. Cryopreserved mesenchymal stromal cells display impaired immunosuppressive properties as a result of heat-shock response and impaired interferon-gamma licensing. *Cytotherapy* **2012**, *14*, 147–152. [\[CrossRef\]](#)
77. Pollock, K.; Sumstad, D.; Kadidlo, D.; McKenna, D.H.; Hubel, A. Clinical mesenchymal stromal cell products undergo functional changes in response to freezing. *Cytotherapy* **2015**, *17*, 38–45. [\[CrossRef\]](#)
78. Galipeau, J. The mesenchymal stromal cells dilemma—does a negative phase III trial of random donor mesenchymal stromal cells in steroid-resistant graft-versus-host disease represent a death knell or a bump in the road? *Cytotherapy* **2013**, *15*, 2–8. [\[CrossRef\]](#)
79. Bahsoun, S.; Coopman, K.; Akam, E.C. Quantitative assessment of the impact of cryopreservation on human bone marrow-derived mesenchymal stem cells: Up to 24 h post-thaw and beyond. *Stem Cell Res. Ther.* **2020**, *11*, 540. [\[CrossRef\]](#)
80. Szablowska-Gadomska, I.; Rudziński, S.; Dymowska, M. Secretome of mesenchymal stromal cells as a possible innovative therapeutic tool in facial nerve injury treatment. *BioMed Res. Int.* **2023**, *2023*, 8427200. [\[CrossRef\]](#)
81. Ceccarelli, S.; Pontecorvi, P.; Anastasiadou, E.; Napoli, C.; Marchese, C. Immunomodulatory Effect of Adipose-Derived Stem Cells: The Cutting Edge of Clinical Application. *Front. Cell Dev. Biol.* **2020**, *8*, 236. [\[CrossRef\]](#)
82. Vizoso, F.J.; Eiro, N.; Cid, S.; Schneider, J.; Perez-Fernandez, R. Mesenchymal stem cell secretome: Toward cell-free therapeutic strategies in regenerative medicine. *Int. J. Mol. Sci.* **2017**, *18*, 1852. [\[CrossRef\]](#) [\[PubMed\]](#)
83. Van Norman, G.A. Limitations of animal studies for predicting toxicity in clinical trials: Is it time to rethink our current approach? *JACC Basic Transl. Sci.* **2019**, *4*, 845–854. [\[CrossRef\]](#) [\[PubMed\]](#)
84. Zhou, J.; Shi, Y. Mesenchymal stem/stromal cells (MSCs): Origin, immune regulation, and clinical applications. *Cell Mol. Immunol.* **2023**, *20*, 555–557. [\[CrossRef\]](#) [\[PubMed\]](#)
85. Mazini, L.; Ezzoubi, M.; Malka, G. Overview of current adipose-derived stem cell (ADSCs) processing involved in therapeutic advancements: Flow chart and regulation updates before and after COVID-19. *Stem Cell Res. Ther.* **2021**, *12*, 1. [\[CrossRef\]](#)
86. Cofano, F.; Boido, M.; Monticelli, M.; Zenga, F.; Ducati, A.; Vercelli, A.; Garbossa, D. Mesenchymal Stem Cells for Spinal Cord Injury: Current Options, Limitations, and Future of Cell Therapy. *Int. J. Mol. Sci.* **2019**, *20*, 2698. [\[CrossRef\]](#)
87. Dixon, J.E.; Shah, D.A.; Rogers, C.; Hall, S.; Weston, N.; Parmenter, C.D.; McNally, D.; Denning, C.; Shakesheff, K.M. Combined hydrogels that switch human pluripotent stem cells from self-renewal to differentiation. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 5580–5585. [\[CrossRef\]](#)



88. Kaisang, L.; Siyu, W.; Lijun, F.; Daoyan, P.; Xian, C.J.; Jie, S. Adipose-derived stem cells seeded in Pluronic F-127 hydrogel promotes diabetic wound healing. *J. Surg. Res.* **2017**, *217*, 63–74. [\[CrossRef\]](#)
89. Nguyen, B.B.; Moriarty, R.A.; Kamalidinov, T.; Etheridge, J.M.; Fisher, J.P. Collagen hydrogel scaffold promotes mesenchymal stem cell and endothelial cell coculture for bone tissue engineering. *J. Biomed. Mater. Res. A* **2017**, *105*, 1123–1131. [\[CrossRef\]](#)
90. Yu, H.; Cheng, J.; Shi, W.; Ren, B.; Zhao, F.; Shi, Y.; Yang, P.; Duan, X.; Zhang, J.; Fu, X.; et al. Bone marrow mesenchymal stem cell-derived exosomes promote tendon regeneration by facilitating the proliferation and migration of endogenous tendon stem/progenitor cells. *Acta Biomater.* **2020**, *106*, 328–341. [\[CrossRef\]](#)
91. Shi, Q.; Qian, Z.; Liu, D.; Sun, J.; Wang, X.; Liu, H.; Xu, J.; Guo, X. GMSC-Derived Exosomes Combined with a Chitosan/Silk Hydrogel Sponge Accelerates Wound Healing in a Diabetic Rat Skin Defect Model. *Front. Physiol.* **2017**, *8*, 904. [\[CrossRef\]](#)
92. Tyeb, S.; Shiekh, P.A.; Verma, V.; Kumar, A. Adipose-Derived Stem Cells (ADSCs) Loaded Gelatin-Sericin-Laminin Cryogels for Tissue Regeneration in Diabetic Wounds. *Biomacromolecules* **2020**, *21*, 294–304. [\[CrossRef\]](#) [\[PubMed\]](#)
93. Wu, Y.Y.; Jiao, Y.P.; Xiao, L.L.; Li, M.M.; Liu, H.W.; Li, S.H.; Liao, X.; Chen, Y.T.; Li, J.X.; Zhang, Y. Experimental Study on Effects of Adipose-Derived Stem Cell-Seeded Silk Fibroin Chitosan Film on Wound Healing of a Diabetic Rat Model. *Ann. Plast. Surg.* **2018**, *80*, 572–580. [\[CrossRef\]](#) [\[PubMed\]](#)
94. Seo, E.; Lim, J.S.; Jun, J.B.; Choi, W.; Hong, I.S.; Jun, H.S. Exendin-4 in combination with adipose-derived stem cells promotes angiogenesis and improves diabetic wound healing. *J. Transl. Med.* **2017**, *15*, 35. [\[CrossRef\]](#) [\[PubMed\]](#)
95. Guillaumat-Prats, R. The Role of MSC in Wound Healing, Scarring and Regeneration. *Cells* **2021**, *10*, 1729. [\[CrossRef\]](#)
96. Rozier, P.; Maumus, M.; Maria, A.T.J.; Toupet, K.; Lai-Kee-Him, J.; Jorgensen, C.; Guilpain, P.; Noel, D. Mesenchymal stromal cells-derived extracellular vesicles alleviate systemic sclerosis via miR-29a-3p. *J. Autoimmun.* **2021**, *121*, 102660. [\[CrossRef\]](#)
97. Yan, J.; Liang, J.; Cao, Y.; El Akkawi, M.M.; Liao, X.; Chen, X.; Li, C.; Li, K.; Xie, G.; Liu, H. Efficacy of topical and systemic transplantation of mesenchymal stem cells in a rat model of diabetic ischemic wounds. *Stem Cell Res. Ther.* **2021**, *12*, 220. [\[CrossRef\]](#)
98. Tanios, E.; Ahmed, T.M.; Shafik, E.A.; Sherif, M.F.; Sayed, D.; Gaber, N.; Hassan, Y. Efficacy of adipose-derived stromal vascular fraction cells in the management of chronic ulcers: A randomized clinical trial. *Regen. Med.* **2021**, *16*, 975–988. [\[CrossRef\]](#)
99. Uzun, E.; Guney, A.; Gonen, Z.B.; Ozkul, Y.; Kafadar, I.H.; Gunay, M.; Mutlu, M. Intraleisional allogeneic adipose-derived stem cells application in chronic diabetic foot ulcer: Phase I/2 safety study. *Foot Ankle Surg.* **2021**, *27*, 636–642. [\[CrossRef\]](#)

**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

## 7. Podsumowanie

Przedstawione w rozprawie doktorskiej publikacje wnoszą istotny wkład w rozwój wiedzy na temat wykorzystania mezenchymalnych komórek macierzystych w leczeniu trudno gojących się ran w przebiegu cukrzycy oraz wpływu statyn i hipoksji na właściwości tych komórek. W ujęciu globalnym badania nad MSC, prekondycjonowaniem komórek i terapią komórkową cieszą się coraz większym zainteresowaniem. Wpisują się również w nowe trendy medycyny regeneracyjnej, dążącej do opracowania skutecznych, bezpiecznych i możliwych do szerokiego zastosowania metod leczenia powikłań cukrzycy.

**Praca nr 1** koncentruje się na ocenie wpływu statyn oraz stężenia tlenu na MSC. Autorzy przeprowadzili serię doświadczeń, w których badano przede wszystkim proliferację oraz żywotność MSC pochodzących ze szpiku kostnego po ekspozycji na różne stężenia atorwastatyny i rosuwastatyny. Jednocześnie komórki hodowano w warunkach normoksji (21% O<sub>2</sub>) i hipoksji (2% O<sub>2</sub>), aby odwzorować możliwe warunki panujące w tkankach niedotlenionych, co jest szczególnie istotne w stanach niedokrwienych (np. w uszkodzonym mięśniu sercowym). Wyniki wskazują, że choć obie statyny w warunkach hipoksji zwiększały indeks proliferacji MSC, to atorwastatyna wykazywała bardziej stabilny profil bezpieczeństwa, z niewielkim efektem cytotoksycznym w porównaniu z rosuwastatyną. Natomiast w warunkach normoksji atorwastatyna istotnie sprzyjała proliferacji komórek, jednocześnie nie zwiększając ich apoptozy. Dodatkowo, nie stwierdzono znaczących różnic w odpowiedzi na statyny pomiędzy grupami komórek pozyskanych od młodszych oraz starszych dawców, co może sugerować podobną zdolność terapeutyczną komórek niezależnie od wieku. Praca ta wnosi istotny wkład w zrozumienie wpływu statyn na MSC, zwłaszcza w warunkach niedotlenienia i w kontekście różnic związanych z wiekiem dawcy.

**Praca nr 2** dotyczy zastosowania allogenicznych ADSC w leczeniu owrzodzeń w zespole stopy cukrzycowej. W badaniu klinicznym pacjentów z zespołem stopy cukrzycowej o etiologii neuropatycznej podzielono na dwie grupy - w jednej grupie zastosowano klej tkankowy niezawierający komórek, w drugiej podano do zmiany troficzej ten sam preparat, lecz z dodatkiem allogenicznych ADSC. Wyniki potwierdziły istotne, statystycznie znaczące przyspieszenie gojenia ran w grupie otrzymującej ADSC. Zaobserwowano krótszy czas do zredukowania rozmiaru rany o 50%, częstsze całkowite zagojenie oraz brak istotnych działań niepożądanych związanych z użyciem allogenicznych



ADSC. Co więcej, autorzy przeprowadzili analizę proteomiczną materiału uzyskanego z łożyska rany, a także potwierdzili obecność komórek dawcy nawet do kilku tygodni po aplikacji. Dzięki temu uchwycono możliwy mechanizm molekularny działania ADSC - przede wszystkim w zakresie modulacji odpowiedzi zapalnej oraz wsparcia procesów angiogenezy. Wnioski te wspierają dalszy rozwój terapii komórkowych w leczeniu trudno gojących się ran, w tym zwłaszcza ran w przebiegu cukrzycy.

**Praca nr 3** stanowi przegląd literatury na temat wykorzystania mezenchymalnych komórek zrębowych w leczeniu ran u pacjentów z cukrzycą, skupiając się w szczególności na owrzodzeniach stopy cukrzycowej. Autorzy omawiają patofizjologiczne aspekty zaburzonego gojenia ran w cukrzycy, w tym roli endoteliopatii, neuropatii i immunopatii, oraz analizują najnowsze wyniki badań wskazujące na skuteczność MSC w terapii komórkowej. W przeglądzie wyróżniono kilka mechanizmów działania MSC, w tym ich zdolność do immunomodulacji (np. hamowanie nadmiernej reakcji zapalnej), wspierania angiogenezy i produkcji czynników wzrostu czy regulacji przebudowy macierzy zewnątrzkomórkowej. Omówiono też nowe strategie mające na celu optymalizację terapii, np. prekondycjonowanie komórek w warunkach hipoksji, inżynierię genetyczną lub łączenie MSC z nośnikami hydrożelowymi. Autorzy wskazują na konieczność prowadzenia dalszych, większych badań klinicznych, niezbędnych do ujednolicenia protokołów podawania MSC i potwierdzenia bezpieczeństwa oraz trwałości efektu terapeutycznego w codziennej praktyce.

## 8. Wnioski

Na podstawie zebranych wyników i przeprowadzonej analizy można wyciągnąć następujące wnioski:

1. Zastosowanie statyn może korzystnie modulować aktywność MSC w warunkach ograniczonej dostępności tlenu. W szczególności atorwastatyna wydaje się wykazywać stabilny profil bezpieczeństwa i zwiększać tempo proliferacji MSC, co podkreśla jej potencjał we wspieraniu terapii opartych na komórkach macierzystych, zwłaszcza w modelach niedotlenienia.
2. ADSC mogą istotnie usprawniać proces gojenia ran w zespole stopy cukrzycowej, co potwierdzono w badaniu klinicznym opisanym w pracy nr 2. Przyspieszone zmniejszanie się obszaru rany oraz większy odsetek całkowitego wygojenia wskazują na potencjał ADSC w terapii trudno gojących się owrzodzeń, przy jednocześnie niskim ryzyku powikłań.
3. Analizy proteomiczne potwierdzają molekularne podstawy korzystnego działania MSC, w tym modulację procesów zapalnych i wsparcie angiogenezy. Obserwowana obecność allogenicznych MSC w łożysku rany przez kilka tygodni po podaniu sugeruje, że komórki te aktywnie uczestniczą w procesie regeneracji tkanek i mogą sprzyjać uruchamianiu lokalnych szlaków naprawczych.
4. Szeroki zakres immunomodulacyjnych, angiogennych i regeneracyjnych właściwości MSC (zarówno w warunkach *in vitro*, jak i *in vivo*) stanowi przesłankę do dalszego rozwijania zaawansowanych terapii komórkowych w leczeniu przewlekłych ran, zwłaszcza w przebiegu cukrzycy. Przegląd dotychczasowych doniesień wskazuje, że łączenie MSC z innowacyjnymi technologiami (np. hydrożelami, modyfikacjami genetycznymi) może dodatkowo poprawiać efektywność terapii.
5. Konieczne jest kontynuowanie badań klinicznych i standaryzacja protokołów terapeutycznych, obejmująca m.in. dobór optymalnego źródła komórek, sposób ich przygotowania i aplikacji, a także ścisłą kontrolę parametrów bezpieczeństwa. Uzyskane dotąd wyniki są obiecujące i otwierają drogę do wprowadzenia terapii z udziałem MSC do rutynowej praktyki klinicznej, jednak kluczowe pozostaje przeprowadzenie dalszych badań z większą liczbą pacjentów i dłuższą obserwacją.

## 9. Bibliografia

1. Sun H, Saeedi P, Karuranga S, Pinkepank M, Ogurtsova K, Duncan BB, et al. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes Res Clin Pract.* 2022;183:109119.
2. Volmer-Thole M, Lobmann R. Neuropathy and Diabetic Foot Syndrome. *Int J Mol Sci.* 2016;17(6).
3. Shi Y, Vanhoutte PM. Macro- and microvascular endothelial dysfunction in diabetes. *J Diabetes.* 2017;9(5):434-49.
4. Bandyk DF. The diabetic foot: Pathophysiology, evaluation, and treatment. *Semin Vasc Surg.* 2018;31(2-4):43-8.
5. Frykberg RG, Banks J. Challenges in the Treatment of Chronic Wounds. *Adv Wound Care (New Rochelle).* 2015;4(9):560-82.
6. Tran HQ, Shahriar SMS, Yan Z, Xie J. Recent Advances in Functional Wound Dressings. *Adv Wound Care (New Rochelle).* 2023;12(7):399-427.
7. Liu J, Gao J, Liang Z, Gao C, Niu Q, Wu F, et al. Mesenchymal stem cells and their microenvironment. *Stem Cell Res Ther.* 2022;13(1):429.
8. Marquez-Curtis LA, Elliott JAW. Mesenchymal stromal cells derived from various tissues: Biological, clinical and cryopreservation aspects: Update from 2015 review. *Cryobiology.* 2024;115:104856.
9. Marfia G, Navone SE, Di Vito C, Ughi N, Tabano S, Miozzo M, et al. Mesenchymal stem cells: potential for therapy and treatment of chronic non-healing skin wounds. *Organogenesis.* 2015;11(4):183-206.
10. Dai G, Xu Q, Luo R, Gao J, Chen H, Deng Y, et al. Atorvastatin treatment improves effects of implanted mesenchymal stem cells: meta-analysis of animal models with acute myocardial infarction. *BMC Cardiovasc Disord.* 2015;15:170.
11. Xu R, Chen J, Cong X, Hu S, Chen X. Lovastatin protects mesenchymal stem cells against hypoxia- and serum deprivation-induced apoptosis by activation of PI3K/Akt and ERK1/2. *J Cell Biochem.* 2008;103(1):256-69.

12. Zhang Q, Yang YJ, Wang H, Dong QT, Wang TJ, Qian HY, et al. Autophagy activation: a novel mechanism of atorvastatin to protect mesenchymal stem cells from hypoxia and serum deprivation via AMP-activated protein kinase/mammalian target of rapamycin pathway. *Stem Cells Dev.* 2012;21(8):1321-32.
13. Song L, Yang YJ, Dong QT, Qian HY, Gao RL, Qiao SB, et al. Atorvastatin enhance efficacy of mesenchymal stem cells treatment for swine myocardial infarction via activation of nitric oxide synthase. *PLoS One.* 2013;8(5):e65702.
14. Hu X, Wu R, Shehadeh LA, Zhou Q, Jiang C, Huang X, et al. Severe hypoxia exerts parallel and cell-specific regulation of gene expression and alternative splicing in human mesenchymal stem cells. *BMC Genomics.* 2014;15:303.
15. Shaked G, Czeiger D, Abu Arar A, Katz T, Harman-Boehm I, Sebbag G. Intermittent cycles of remote ischemic preconditioning augment diabetic foot ulcer healing. *Wound Repair Regen.* 2015;23(2):191-6.



## 10. Oświadczenia współautorów

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Agnieszka Kulesza  
(imię i nazwisko)

### OŚWIADCZENIE

Jako współautor pracy pt. **The influence of oxygen deprivation and donor age on the effect of statins on human mesenchymal stromal cells**. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

przeprowadzenie badania, przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 4 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 30 %,

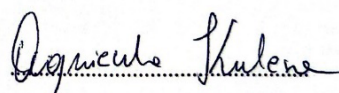
(imię i nazwisko kandydata do stopnia)

obejmował on: pozyskanie finansowania, opracowanie koncepcji i metodyki pracy, przeprowadzenie badania, interpretacja wyników, przygotowanie manuskryptu.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)

  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Leszek Czupryniak  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opracowanie koncepcji i metodyki pracy, umożliwienie przeprowadzenia badania, opieka merytoryczna.

Mój udział procentowy w przygotowaniu publikacji określam jako 2,5 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 3 %,

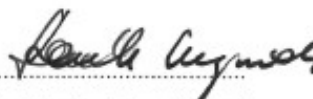
(imię i nazwisko kandydata do stopnia)

obejmował on: przeprowadzenie badania i przygotowanie manuskryptu

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)

  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

---

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Leszek Czupryniak  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **The Potential of Mesenchymal Stem/Stromal Cells in Diabetic Wounds and Future Directions for Research and Therapy—Is It Time for Use in Everyday Practice?** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opieka merytoryczna.

Mój udział procentowy w przygotowaniu publikacji określam jako 2,5 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 50 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: opracowanie konceptu i metodyki pracy, analizę danych, przygotowanie manuskryptu.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)



(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Anna Nowak-Szwed  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **The Potential of Mesenchymal Stem/Stromal Cells in Diabetic Wounds and Future Directions for Research and Therapy—Is It Time for Use in Everyday Practice?** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 4 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 50 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: opracowanie konceptu i metodyki pracy, analizę danych, przygotowanie manuskryptu.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)



(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników



Warszawa, 24.03.2025 r.  
(miejscowość, data)

Ilona Szablowska-Gadomska  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **The Potential of Mesenchymal Stem/Stromal Cells in Diabetic Wounds and Future Directions for Research and Therapy—Is It Time for Use in Everyday Practice?** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opracowanie konceptu pracy i przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 12 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 50 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: opracowanie konceptu i metodyki pracy, analizę danych, przygotowanie manuskryptu.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)

*Ilona Szablowska-Gadomska*

(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Ilona Szablowska-Gadomska  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

pozyskanie finansowania, opracowanie koncepcji i metodyki pracy, umożliwienie przeprowadzenia badania, analiza danych, przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określłam jako 25 %.

Wkład Damian Sieńko w powstawanie publikacji określłam jako 3 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: przeprowadzenie badania i przygotowanie manuskryptu

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)

*Ilona Szablowska-Gadomska*  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Stefan Rudziński  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opracowanie koncepcji i metodyki pracy, przeprowadzenie badania, analiza danych, przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 5 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 3 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: przeprowadzenie badania i przygotowanie manuskryptu

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)



(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Stefan Rudziński  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **The Potential of Mesenchymal Stem/Stromal Cells in Diabetic Wounds and Future Directions for Research and Therapy—Is It Time for Use in Everyday Practice?** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

przygotowanie manuskryptu oraz wizualizacja danych.

Mój udział procentowy w przygotowaniu publikacji określam jako 4 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 50 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: opracowanie konceptu i metodyki pracy, analizę danych, przygotowanie manuskryptu.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)



(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników



Warszawa, 24.03.2025 r.  
(miejscowość, data)

Rafał Płoski  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opracowanie koncepcji i metodyki pracy, przeprowadzenie badania, analiza danych, przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 4 %.

Wkład Damian Sienko w powstawanie publikacji określam jako 3 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: przeprowadzenie badania i przygotowanie manuskryptu

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sienko.

(imię i nazwisko kandydata do stopnia)

  
.....  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Piotr Gasperowicz  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opracowanie metodyki pracy, analiza danych, przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 2,5 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 3 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: przeprowadzenie badania i przygotowanie manuskryptu

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)

  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Beata Mrozikiewicz-Rakowska  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **The Potential of Mesenchymal Stem/Stromal Cells in Diabetic Wounds and Future Directions for Research and Therapy—Is It Time for Use in Everyday Practice?** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opracowanie koncepcji i metodyki pracy, przygotowanie manuskryptu, opieka merytoryczna.

Mój udział procentowy w przygotowaniu publikacji określam jako 12 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 50 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: opracowanie konceptu i metodyki pracy, analizę danych, przygotowanie manuskryptu.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)



(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Beata Mrozikiewicz-Rakowska  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opracowanie koncepcji i metodyki pracy, umożliwienie przeprowadzenia badania, analiza danych, przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 25 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 3 %,

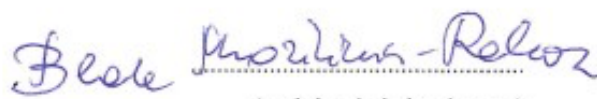
(imię i nazwisko kandydata do stopnia)

obejmował on: przeprowadzenie badania i przygotowanie manuskryptu

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)

  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników



Warszawa, 24.03.2025 r.  
(miejscowość, data)

Marek Kuch  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **The influence of oxygen deprivation and donor age on the effect of statins on human mesenchymal stromal cells**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

przygotowanie manuskryptu i wsparcie merytoryczne.

Mój udział procentowy w przygotowaniu publikacji określam jako 4 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 30 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: pozyskanie finansowania, opracowanie koncepcji i metodyki pracy, przeprowadzenie badania, interpretacja wyników, przygotowanie manuskryptu.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)

  
.....  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Tomasz Grzela  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opracowanie koncepcji i metodyki pracy, analiza danych i przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określłam jako 3 %.

Wkład Damian Sienko w powstawanie publikacji określłam jako 3 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: przeprowadzenie badania i przygotowanie manuskryptu

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sienko.

(imię i nazwisko kandydata do stopnia)

dr hab. n. med. Tomasz Grzela  
Specjalista chirurgii ogólnej  
1800017  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Mateusz Mieczkowski  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

przeprowadzenie badania i przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określłam jako 3 %.

Wkład Damian Sieńko w powstawanie publikacji określłam jako 3 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: przeprowadzenie badania i przygotowanie manuskryptu

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)

.....Mateusz Mieczkowski

(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Anna Burdzińska  
(imię i nazwisko)

### OŚWIADCZENIE

Jako współautor pracy pt. **The influence of oxygen deprivation and donor age on the effect of statins on human mesenchymal stromal cells**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opracowanie koncepcji i metodyki pracy, przeprowadzenie badania, interpretacja wyników, wizualizacja wyników, przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 25 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 30 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: pozyskanie finansowania, opracowanie koncepcji i metodyki pracy, przeprowadzenie badania, interpretacja wyników, przygotowanie manuskryptu.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)

  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników



Warszawa, 24.03.2025 r.  
(miejscowość, data)

Dominika Klimczak-Tomaniak  
(imię i nazwisko)

### OŚWIADCZENIE

Jako współautor pracy pt. **The influence of oxygen deprivation and donor age on the effect of statins on human mesenchymal stromal cells**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opracowanie koncepcji i metodyki pracy, przeprowadzenie badania, interpretacja wyników, przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 30 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 30 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: pozyskanie finansowania, opracowanie koncepcji i metodyki pracy, przeprowadzenie badania, interpretacja wyników, przygotowanie manuskryptu.

\*\*\*

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)

dr hab. n. med.  
Dominika Klimczak-Tomaniak  
specjalista chorób wewnętrznych  
2961033

*Dominika Klimczak-Tomaniak*

(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Małgorzata Lewandowska-Szumiel  
(imię i nazwisko)

### OŚWIADCZENIE

Jako współautor pracy pt. **The Potential of Mesenchymal Stem/Stromal Cells in Diabetic Wounds and Future Directions for Research and Therapy—Is It Time for Use in Everyday Practice?** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

przygotowanie manuskryptu, opieka merytoryczna.

Mój udział procentowy w przygotowaniu publikacji określłam jako 4 %.

Wkład Damian Sieńko w powstawanie publikacji określłam jako 50 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: opracowanie konceptu i metodyki pracy, analizę danych, przygotowanie manuskryptu.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)

  
.....  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Maksymilian Gofron  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **The Potential of Mesenchymal Stem/Stromal Cells in Diabetic Wounds and Future Directions for Research and Therapy—Is It Time for Use in Everyday Practice?** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 4 %.

Wkład **Damian Sieńko** w powstawanie publikacji określam jako 50 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: opracowanie konceptu i metodyki pracy, analizę danych, przygotowanie manuskryptu.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. **Damiana Sieńko**.

(imię i nazwisko kandydata do stopnia)

*Maksymilian Gofron*

(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Przemysław Zygmunciak  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **The Potential of Mesenchymal Stem/Stromal Cells in Diabetic Wounds and Future Directions for Research and Therapy—Is It Time for Use in Everyday Practice?** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

wizualizacja danych.

Mój udział procentowy w przygotowaniu publikacji określam jako 2 %.

Wkład Damian Sienko w powstawanie publikacji określam jako 50 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: opracowanie konceptu i metodyki pracy, analizę danych, przygotowanie manuskryptu.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sienko.

(imię i nazwisko kandydata do stopnia)

.....  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników



Warszawa, 24.03.2025 r.  
(miejscowość, data)

Wojciech Stanisław Zgliczyński  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **The Potential of Mesenchymal Stem/Stromal Cells in Diabetic Wounds and Future Directions for Research and Therapy—Is It Time for Use in Everyday Practice?** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opieka merytoryczna.

Mój udział procentowy w przygotowaniu publikacji określam jako 2,5 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 50 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: opracowanie konceptu i metodyki pracy, analizę danych, przygotowanie manuskryptu.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)

KIEROWNIK  
KLINIKI ENDOKRYNOLOGII CMKP  
  
Prof. dr hab. n. med. Wojciech Zgliczyński  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Barbara Paleska  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

analiza i wizualizacja danych, przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 3 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 3 %,

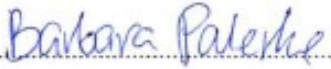
(imię i nazwisko kandydata do stopnia)

obejmował on: przeprowadzenie badania i przygotowanie manuskryptu

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)

  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Dominik Cysewski  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opracowanie koncepcji i metodyki pracy, przeprowadzenie badania, analiza danych, przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 10 %.

Wkład Damian Sienko w powstawanie publikacji określam jako 3 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: przeprowadzenie badania i przygotowanie manuskryptu

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sienko.

(imię i nazwisko kandydata do stopnia)

  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Magdalena Konarzewska  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact**. oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opracowanie metodyki pracy, analiza danych, przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określłam jako 3 %.

Wkład Damian Sieńko w powstawanie publikacji określłam jako 3 %,

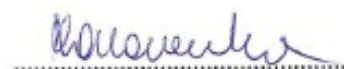
(imię i nazwisko kandydata do stopnia)

obejmował on: przeprowadzenie badania i przygotowanie manuskryptu

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)



(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników



Warszawa, 24.03.2025 r.  
(miejscowość, data)

Aleksandra Helena Symonides-Pomianek  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. *The influence of oxygen deprivation and donor age on the effect of statins on human mesenchymal stromal cells*, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

przeprowadzenie badania, przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 3 %.

Wkład Damian Sienko w powstawanie publikacji określam jako 30 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: pozyskanie finansowania, opracowanie koncepcji i metodyki pracy, przeprowadzenie badania, interpretacja wyników, przygotowanie manuskryptu.

(merytoryczny opis wkładu kandydata do stopnia w powstaniu publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sienko.

(imię i nazwisko kandydata do stopnia)

*Aleksandra Symonides-Pomianek*  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Małgorzata Lewandowska-Szumieł  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

ustalenia koncepcji i metodyki pracy, pozyskanie finansowania, umożliwienie wykonania badania, analiza danych, przygotowanie manuskryptu, opieka merytoryczna.

Mój udział procentowy w przygotowaniu publikacji określam jako 5 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 3 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: przeprowadzenie badania i przygotowanie manuskryptu

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)



(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.  
(miejscowość, data)

Jakub Zieliński  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opracowanie metodyki pracy, analiza danych, przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 3 %.

Wkład Damian Sienko w powstawanie publikacji określam jako 3 %,

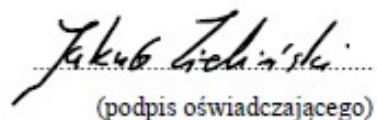
(imię i nazwisko kandydata do stopnia)

obejmował on: przeprowadzenie badania i przygotowanie manuskryptu

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sienko.

(imię i nazwisko kandydata do stopnia)

  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 24.03.2025 r.

Prof. dr hab. med. Leszek Pączek

### OŚWIADCZENIE

Jako współautor pracy pt. **The influence of oxygen deprivation and donor age on the effect of statins on human mesenchymal stromal cells**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

umożliwienie wykonania badania, przygotowanie manuskryptu i wsparcie merytoryczne.

Mój udział procentowy w przygotowaniu publikacji określam jako 4 %.

Wkład Damian Sienko w powstawanie publikacji określam jako 30 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: pozyskanie finansowania, opracowanie koncepcji i metodyki pracy, przeprowadzenie badania, interpretacja wyników, przygotowanie manuskryptu.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sienko.

(imię i nazwisko kandydata do stopnia)



(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników



Warszawa, 24.03.2025 r.  
(miejscowość, data)

Maria Noszczyk  
(imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. **Allogenic Adipose-Derived Stem Cells in Diabetic Foot Ulcer Treatment: Clinical Effectiveness, Safety, Survival in the Wound Site, and Proteomic Impact**, oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

opracowanie koncepcji i metodyki pracy, analiza danych i przygotowanie manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 3 %.

Wkład Damian Sieńko w powstawanie publikacji określam jako 3 %,

(imię i nazwisko kandydata do stopnia)

obejmował on: przeprowadzenie badania i przygotowanie manuskryptu

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)\*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Damiana Sieńko.

(imię i nazwisko kandydata do stopnia)

Maria Noszczyk  
(podpis oświadczającego)

\*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników