lek. Magdalena Nita

„Ocena bezpieczeństwa i skuteczności opatrunku biologicznego w chirurgicznym leczeniu ran”

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

Promotor: prof. dr hab. Piotr Fiedor

Klinika Chirurgii Ogólnej i Transplantacyjnej UCK WUM Szpital Kliniczny Dzieciątka Jezus

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1. *„**Review of the Latest Methods of Epidermolysis Bullosa and Other Chronic Wounds Treatment Including BIOOPA Dressing***" (tłum. „*Przegląd najnowszych metod leczenia Epidermolysis Bullosa i innych ran przewlekłych włączając opatrunek BIOOPA*") Dermatology and Therapy, doi: 10.1007/s13555-021-00578-w.
2. Rozdział książki „Rare diseases" (tłumaczenie: „Choroby rzadkie") opublikowany przez wydawnictwo Intechopen pt. ***„Surgical treatment of wound dressing stem cells in Epidermolysis Bullosa (EB)"*** (*tłum. „Chirurgiczne leczenie ran w Epidermolysis Bullosa opatrunkiem z komórkami macierzystymi*"), doi:10.5772/intechopen.97036.
3. *„**New Treatment of Wound Healing With Allogenic Acellular Human Skin Graft: Preclinical Assessment and In Vitro Study**"* (tłum. *„Nowa metoda leczenia ran przy użyciu allogenicznego, acelularnego przeszczepu skóry - badanie przedkliniczne i badanie in vitro*"); Transplantation Proceedings, doi:10.1016/j.transproceed.2020.02.115.
4. ***„Transplantation of a New Biological Product in Rare Diseases, Such as Epidermolysis Bullosa: Response and Clinical Outcome"*** (tłum. „*Przeszczepienie nowego produktu biologicznego w chorobach rzadkich, takich jak Epidermolysis Bullosa: odpowiedź i skuteczność kliniczna*"); Transplantation Proceedings, doi:10.1016/ j.transproceed. 2020.02.119.

Spis treści

Wykaz publikacji stanowiących pracę doktorską 4

Wykaz stosowanych skrótów 6

Streszczenie w języku polskim 7

Streszczenie w języku angielskim 8

Rozdział I Wstęp 9

Rozdział II Założenia i cel pracy 14

Rozdział III Kopie opublikowanych prac 22

Rozdział IV Podsumowanie wyników badań i wnioski końcowe 67

Załączniki 68

Oświadczenie Komisji Bioetycznej przy Warszawskim Uniwersytecie Medycznym *... 68*

Oświadczenia współautorów publikacji *..... 70*

Literatura 104

Wykaz stosowanych skrótów

ASCT – przeszczep allogenicznych komórek macierzystych (ang. *allogenic stem cell transplantation*)

ATMP – produkt medyczny terapii zaawansowanej (ang. *advanced therapy medicinal product*)

BMT – przeszczep szpiku kostnego (ang. *bone marrow transplant*)

CVD – przewlekła choroba żylna (ang. *chronic venous disease*)

EB – pęcherzowe oddzielanie się naskórka (łac. *Epidermolysis Bullosa*)

RDEB – recesywna, dystroficzna postać *Epidermolysis Bullosa* (ang. *recessive dystrophic Epidermolysis Bullosa*)

TGF – transformujący czynnik wzrostu (ang. *transforming growth factor*)

WJ – galareta Whortona (ang. *Whorhon jelly*)

WJ-MSC – komórki mezenchymalne z galarety Whortona (ang. *Whorhon jelly mesenchymal stem cells*)

Streszczenie w języku polskim

Trudno gojące się rany to istotny problem kliniczny i wyzwanie w praktyce lekarza. Najczęściej powstają na skutek niewydolności żylnej, zaburzeń ukrwienia, powikłań cukrzycy, oparzeń, jak również w przebiegu chorób rzadkich takich jak pęcherzykowe oddzielanie się naskórka (*Epidermolysis Bullosa*, EB). W niniejszej pracy przedstawiono opatrunek biologiczny w postaci acelularnej, allogenicznej skóry, sterylizowanej radiacyjnie i zasiedlonej komórkami macierzystymi z galarety Whortona. Opatrunek ten klasyfikowany jako produkt terapii zaawansowanej (*advanced therapy medical product*, ATMP), który stanowi nadzieję dla pacjentów z ranami przewlekłymi różnego pochodzenia.

W skład rozprawy wchodzą cztery prace: jeden rozdział książki i trzy artykuły tworzące cykl publikacji. Są one cennym źródłem informacji na temat bezpieczeństwa i skuteczności opatrunków biologicznych w leczeniu ran przewlekłych. Zostały w nich przedstawione wyniki dotyczące użycia bezkomórkowej, allogenicznej skóry pobranej ze zwłok, zasiedlonej komórkami macierzystymi w leczeniu ran przewlekłych na przykładzie pacjentów z EB.

Badania immunohistochemiczne, histologiczne, mikroskopia elektronowa i konfokalna wykazały naciek komórek gospodarza i neowaskularyzację opatrunku biologicznego. Ponadto takie opatrunki charakteryzowały się niską immunogennością, potwierdzoną badaniami histologicznymi i proliferacją limfocytów T *in vitro*. Obserwowano zagojenie się lub zmniejszenie powierzchni rany w okresie obserwacji, jak również redukcję dolegliwości i świądu wśród ochotników biorących udział w badaniu.

Uzyskane wyniki świadczą o skuteczności opatrunku biologicznego w postaci acelularnej, allogenicznej skóry zasiedlonej komórkami macierzystymi w leczeniu ran powstających w przebiegu EB. Dalsze badania nad opatrunkiem biologicznym wśród pacjentów z przewlekłymi owrzodzeniami o różnej etiologii mogą przyczynić się do udoskonalenia chirurgicznego leczenia trudno gojących się ran i polepszenia jakości życia pacjentów.

Streszczenie w języku angielskim

Difficult-to-heal wounds are a significant clinical problem and a challenge in medical practice. Most often wounds arise because of venous insufficiency, blood circulation disorders, diabetes complications, burns, as well as in the course of rare diseases such as *Epidermolysis Bullosa* (EB). The presented study presents a biological dressing in the form of acellular, allogeneic, radiation sterilized skin colonized with stem cells derived from Whorton's jelly. This dressing, classified as an advance medical product (ATMP), offers hope to patients with chronic wounds of various etiology.

The dissertation consists of four publications, one chapter of the book, and three articles that make up the series of publications. They assess the safety and effectiveness of biological dressings in the treatment of chronic wounds. They present the results concerning the use of acellular allogeneic skin collected from the deceased donor, colonized with stem cells in the treatment of chronic wounds, based on the example of patients with EB.

Immunohistochemical and histological examinations as well as electron and confocal microscopy showed infiltration of the host cells and neovascularization of the biological dressing. Moreover, the dressings were characterized by low immunogenicity, confirmed by histological tests and the proliferation of T lymphocytes *in vitro*. Healing or reduction of the wound area was observed during the follow-up period, as well as a reduction in pain and itching among the patients in the study.

The obtained results prove the effectiveness of biological dressing in the form of acellular, allogeneic skin inhabited by stem cells in the treatment of wounds in EB. Further research on biological dressing among patients with chronic wounds of various etiologies may contribute to the improvement of surgical treatment of difficult-to-heal wounds and the improvement of the patients' quality of life.

Rozdział I Wstęp

Wprowadzenie antybiotykoterapii, środków odkażających oraz nowoczesnych opatrunków do codziennej terapii udoskonaliło opiekę, gojenie się ran oraz wydłużyło jakość i komfort życia chorych z przewlekłymi ranami. W szczególności problem ten dotyczy chorych powyżej szóstej dekady życia, którzy ze względu na dolegliwości związane z chorobą nie są i/lub nie mogą być aktywni zawodowo.

Rany przewlekłe, które są przyczyną rosnącej liczby świadczeń socjalnych stanowią ogromny problem ekonomiczny, z którym borykają się państwa rozwinięte i rozwijające się na całym świecie. Za zasadniczą przyczynę uważa się starzejące się społeczeństwo. Rosnąca potrzeba zaawansowanych form opieki medycznej wpływa na gospodarkę. Według raportu *Global Burden of Disease* liczba przypadków ran przewlekłych-chronicznych gwałtownie rośnie, na podstawie zebranych danych oszacowano, że w 2015 problem dotyczył ponad 605 milionów pacjentów [1]. Trudnością w określeniu skali problemu jest brak ściśle określonej, spójnej klasyfikacji umożliwiającej usystematyzowanie wszystkich przyczyn ran chronicznych i definicji charakteryzującej „chroniczność" danej rany. Większość autorów przyjmuje, że za ranę przewlekłą uważa się ubytek skóry spowodowany procesem chorobowym lub urazem, którego czas trwania przekracza 6-8 tygodni [2]. Z powodu nieprecyzyjnej definicji, w wielu przypadkach trudno jednoznacznie sklasyfikować ranę. W różnych rejonach świata wskaźnik chorobowości waha się od 0,168 przypadków na 1000 populacji u kobiet z Ameryki Środkowej do 2,324 przypadków na 1000 populacji u kobiet z Afryki Północnej i Bliskiego Wschodu [3].

Na powstawanie ran przewlekłych wpływ ma wiele czynników, które można podzielić na lokalne/miejscowe i ogólnoustrojowe. Do czynników lokalnych zalicza się między innymi urazy, niedokrwienie, martwicę, ciała obce, nieprawidłową przebudowę macierzy pozakomórkowej, dysfunkcję fibroblastów, zakażenia czy brak odpowiedzi na czynniki wzrostu. Czynniki ogólnoustrojowe obejmują złe odżywianie, niedokrwistość, choroby autoimmunologiczne, choroby naczyń obwodowych, cukrzycę, stosowanie leków immunosupresyjnych i przeciwzapalnych [4,5].

Najczęstszą przyczyną ran chronicznych są owrzodzenia żylne, które stanowią aż 75% wszystkich przypadków. Są one najczęstszą postacią przewlekłej choroby żylnej (*chronic venous disease,* CVD), która w populacji europejskiej dotyka 40%-60% kobiet i 15%-30% mężczyzn [6,7]. Przewlekła niewydolność żylna spowodowana jest

niewydolnością aparatu zastawkowego, co powoduje odpływ krwi żylnej z kończyn [8]. W początkowej fazie CVD objawia się uczuciem „ciężkości nóg" i obrzękami. W kolejnych etapach pojawiają się teleangiektazje, żylaki i niegojące się zmiany troficzne, które w znaczący sposób pogarszają jakość życia pacjentów. Zaproponowana w 1994 roku na Konferencji Amerykańskiego Forum Żylnego klasyfikacja CEAP (*clinical, etiological, anatomical, pathophysiological*) pozwoliła na usystematyzowanie, klasyfikację i monitorowanie leczenia przewlekłej choroby żylnej. Czynne owrzodzenia żylne, będące obiektem badań opisywane są w tej skali jako C-6 [9].

Kolejną co do częstości przyczyną występowania przewlekłych ran są zmiany niedokrwienne (14%) na tle zmian miażdżycowych tętnic kończyn dolnych. Stanowią one aż 98% zmian niedokrwiennych. Kolejną grupą są owrzodzenia w przebiegu stopy cukrzycowej (5%), które powstają na skutek mikro i makroangiopatii. Znaczącym problem jest również neuropatia cukrzycowa, która poprzez zaburzenia odczuwania bólu przyczynia się do zwiększonej ilości urazów. Kolejnym rodzajem ran uwzględnionych w klasyfikacji są owrzodzenia mieszane (o etiologii żylno-tętniczej). Do pozostałych przyczyn, które stanowią < 1% zaliczamy rany przewlekłe powstałe na skutek choroby nowotworowej, chorób autoimmunologicznych, owrzodzenia w przebiegu wrodzonych malformacji naczyniowych oraz chorób o podłożu genetycznym należących do grupy chorób rzadkich takich jak pęcherzykowe oddzielanie się naskórka (*Epidermolysis Bullosa*, EB). Jest to choroba o ciężkim klinicznym przebiegu u noworodków/dzieci i dorosłych obejmująca liczne otwarte, bardzo bolesne rany skóry oraz zmiany wywołane chorobą w innych narządach jak przewód pokarmowy powodujący niedożywienie i wyniszczenie organizmu często prowadzące do śmierci.

Badania naukowe będące podstawą niniejszej pracy doktorskiej dotyczą rozwoju produkt medycznego terapii zaawansowanej (*Advance Medicinal Product,* ATMP) na potrzeby leczenia chirurgicznego ran przewlekłych w EB oraz owrzodzeń troficznych kończyn dolnych u chorych z niewydolnością naczyniową. Wyniki prac naukowych zostały systematycznie opublikowane w celu wykazania bezpieczeństwa i skuteczności terapeutycznej badanego i innowacyjnego opatrunku opracowanego dla tej grupy chorych. Wszystkie badania przedkliniczne i kliniczne zostały wykonane za zgodą Komisji Bioetycznej oraz uzyskały pozytywną decyzję Urząd Rejestracji Produktów Leczniczych na prowadzenia badania klinicznego (BIOOPA DBL.474.317.2020 KB/2019 14.01.2019; KB/177/2015) w ramach projektu badawczego finansowanego z środków publicznych o akronimie BIOOPA.

EB jest przyczyną poszukiwania skutecznych sposobów leczenia ran przewlekłych i jednocześnie stała się tematem i inspiracją powstania niniejszej rozprawy doktorskiej. Zasadniczym problemem pacjentów z EB jest kruchość skóry i tworzące się pęcherze na skórze, powstające na skutek nawet niewielkiego urazu mechanicznego. Do charakterystycznych zmian obok pęcherzy należy zaliczyć prosaki, czyli milie, przebarwienia pigmentacyjne, nadżerki, jak również blizny po wygojonych zmianach. Pęcherze, a w konsekwencji trudno gojące się rany w okolicy stóp i dłoni, które są najbardziej narażone na urazy mechaniczne prowadzą do powstawania przykurczów i zrostów (pseudosyndaktylii). W wielu przypadkach dochodzi do trwałego ubytku naskórka, włączając w to wrodzony brak naskórka. Dodatkowo obserwuje się charakterystyczne zmiany w obrębie płytek paznokciowych obejmujące zarówno dystrofię, jak i brak paznokci. Bliznowaceniu może towarzyszyć dotkliwy świąd. Objawy skórne nie są jedyną konsekwencją EB. Zmiany pojawiają się także wewnątrz ciała powodują powstanie pęcherzy, nadżerek, blizn i zrostów prowadzących do przewężeń, także w obrębie przewodu pokarmowego, dróg moczowych i oddechowych. Rany w przebiegu EB mają tendencję do zakażeń oraz tendencję do rozwoju raka kolczysto-komórkowego. Na skuteczność terapii w dużej mierze wpływ mają dwa czynniki: właściwe leczenie ran i stan ogólny pacjenta [10].

W celu poszukiwania nowych/innowacyjnych metod leczenia ran przewlekłych i analizy ich bezpieczeństwa oraz oceny skuteczności terapeutycznej został opracowany własny materiał kliniczny w odniesieniu do opublikowanych, alternatywnych metod przez wiodące ośrodki kliniczne/uniwersyteckie zebrany w opublikowanym artykule pt. **„*Review of the Latest Methods of Epidermolysis Bullosa and Other Chronic Wounds Treatment Including BIOOPA Dressing*”** (tłum. „*Przegląd najnowszych metod leczenia Epidermolysis Bullosa i innych ran przewlekłych włączając opatrunek BIOOPA*”). Dermatology and Therapy, doi: 10.1007/s13555-021-00578-w. Obecnie nie jest dostępne skuteczne leczenie przyczynowego EB, wyniki stosowania sterydoterapii ogólnoustrojowej, leków przeciwmalarycznych, fotochemioterapii z użyciem psoralenu, czy tetracyklin nie przyniosły zadowalających rezultatów. Leczenie EB opiera się więc na miejscowym zaopatrywaniu ran i leczeniu objawowym.

Istotną rolę odgrywa tryb życia pacjenta, zapewniający ochronę przed ewentualnymi urazami i kontuzjami. W przypadku powikłań takich jak przykurcze i zrosty kończyn w wyniku bliznowacenia złotym standardem pozostaje chirurgia plastyczna. Autogeniczny przeszczep skóry w celu zaopatrzenia niegojących się ran ze względu na

patomechanizm EB nie jest metodą z wyboru. Prowadzi to do zaburzenia gojenia się miejsc z których przeszczep został pobrany i powstawania kolejnych urazów przewlekłych [11]. Do niekorzystnych czynników utrudniających leczenie każdej przewlekłej rany, w tym również urazów w przebiegu EB należy zaliczyć niedożywienie, anemię, jak również zakażenia ran przewlekłych, które prowadzić mogą do ciężkich powikłań. Dlatego tak ważny jest dobór odpowiedniego opatrunku. Według Goertz'a i wsp. skuteczny opatrunek powinien być antybakteryjny, chłonny i nieadhezyjny [12]. Na rynku są dostępne produkty posiadające te cechy. Należą do nich zastygające żele, które początkowo mają płynną konsystencję a po nałożeniu na ranę twardnieją zapewniając optymalne środowisko do leczenia, a także opatrunki na bazie dwuwarstwowej żelatyny jedwabnej, które przyśpieszają gojenie. Innym rodzajem są opatrunki na bazie srebra i chlorheksydyny działające bakteriostatycznie, czy proszek Catrix (Cranage Healthcare International), który składa się z chrząstki bydlęcej, mukopolisacharydów i kolagenu typu II stosowany w leczeniu odleżyn, oparzeń, owrzodzeń i otarć. Badania opisują korzystny wpływ tego opatrunku w leczeniu ran w EB [13].

Mimo całej gamy dostępnych nowoczesnych produktów, nie został wynaleziony idealny opatrunek zaspokajający wszystkie potrzeby pacjenta, dlatego wielką nadzieję pokłada się w inżynierii tkankowej i terapii kombinatorycznej. Inżynieria tkankowa pozwala na wyprodukowanie substytutów w postaci naskórkowej, skórnej i dermoepidermalnej. Składają się one z komórkowej lub bezkomórkowej macierzy, która stanowi trójwymiarowe środowisko do zasiedlenia go wyhodowanymi w środowisku *in vitro* komórkami macierzystymi, fibroblastami, keratynocytami czy komórkami Langerhansa. Po nałożeniu na ranę opatrunki produkują białka macierzy, czynniki wzrostu, które odgrywają zasadniczą rolę w procesie gojenia. Zalicza się do nich takie opatrunki jak Apligraf, który zbudowany jest z dwuwarstwowej matrycy z kolagenu bydlęcego zasiedlonej keratynocytami i fibroblastami pochodzącymi z napletka noworodkowego. Biobrane, nylonowa trójwymiarowa siateczka, w której zawieszone są cząsteczki kolagenu typu I pochodzącego od świń. Całość pokryta jest ultracienką półprzepuszczalną warstwą silikonu, która spełnia rolę naskórka zapobiegającej utracie wody. OrCelTM to dwuwarstwowy kompozyt zbudowany z bydlęcego kolagenu typu I zasiedlonego keratynocytami i fibroblastami. Produkowane przez nie czynniki wzrostu TGF-alfa, czynnik wzrostu keratynocytów-1 i czynnik wzrostu fibroblastów-1 stymulują do gojenia się rany. Produkty inżynierii tkankowej stanowią ogromną nadzieję w leczeniu dużych ran powstałych między innymi na skutek rozległych oparzeń, genodermatoz (EB), gdzie

autogeniczny przeszczep skóry jest niemożliwy. Mimo ogromnego postępu w tej dziedzinie nie udało się wyprodukować substytutu skóry, który spełniałby wszystkie jej funkcje takie jak unaczynienie, termoregulacja, czy prawidłowa pigmentacja [14-22].

W kolejnej publikacji naukowej, w rozdziale książki „Rare diseases" (tłumaczenie: „Choroby rzadkie") opublikowanym przez wydawnictwo Intechopen pod tytułem **„*Surgical treatment of wound dressing stem cells in Epidermolysis Bullosa (EB)*"** (tłum. „*Chirurgiczne leczenie ran w Epidermolysis Bullosa opatrunkiem z komórkami macierzystymi*"), doi:10.5772/intechopen.97036. zostały przedstawione i omówione ATMP. Do tej grupy zaliczają się produkty lecznicze i leki, które opierają się na genach, komórkach lub tkankach. Nowoczesne terapie oferują nowe możliwości leczenia chorób i urazów. ATMP można podzielić na trzy główne typy: leki terapii genowej, leki somatycznej terapii komórkowej i leki inżynierii tkankowej. Ponadto produkty terapii zaawansowanej mogą być stworzone na zasadzie terapii kombinatorycznej, która łączy wyżej wymienione typy ATMP. Przykładem mogą być substytuty skóry, w których komórki z hodowli takie jak komórki macierzyste, fibroblasty, keratynocyty osadzone są w biodegradowalnej matrycy lub rusztowaniu.

Jak dotąd nie jest dostępna terapia przyczynowa odpowiednia dla wszystkich pacjentów z EB. Trwają badania nad terapią genową i komórkową. Opierają się one na hodowli keratynocytów pacjentów z recesywną, dystroficzną postacią EB (RDEB), które zostały transdukowane wektorem retrowirusowym zawierającym pełnej długości cDNA genu *COL7A1* (kodującego kolagen typ VII). Następnie zostały one wprowadzone na rany pacjenta jako płaty naskórka. Wykazano skuteczność i ekspresję kolagenu VII, jednak odpowiedź utrzymywała się do 12 miesięcy. Minusem takiej terapii jest ograniczony obszar jej stosowania (w miejscu występowania ran przewlekłych).

Ponadto dostępne są doniesienia o możliwości wykonania przeszczepienia szpiku i allogenicznych komórek macierzystych (*bone marrow transplant*, BMT *oraz allogenic stem cell transplantation*, ASCT). W 2010 Wagner i wsp. wykonali ASCT u dzieci RDEB, pomimo tego, że nie doszło do wyleczenia, wykazano redukcję pęcherzy na skórze i szybszą regenerację, niestety mimo obiecujących wyników, badania wciąż są na wczesnym etapie i wymagają wnikliwej analizy. Opublikowany rozdział powstał, pomimo że choroby rzadkie to problem dotyczący niewielkiego odsetka społeczeństwa. Globalnie szacuje się, że ok. 350 milionów ludzi cierpi na zaburzenia genetyczne wywołujące choroby rzadkie. Do tej pory odkryto ich ok 7-8 tys. Celem autorów książki było zgromadzenie informacji,

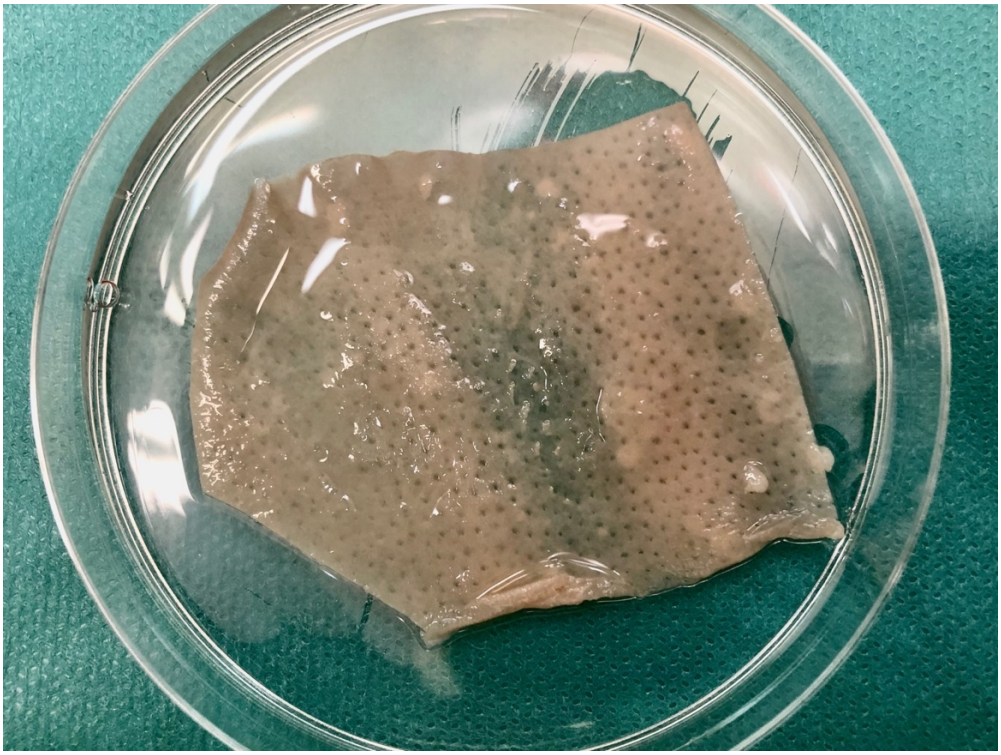
wiedzy i doświadczeń klinicznych autorów w leczeniu i diagnostyce chorób rzadkich, które będą pomocne dla lekarzy, pacjentów i ich rodzin w opracowaniu diagnozy i skutecznych form leczenia.

Na podstawie przeprowadzonej analizy dostępnych form leczenia omówionych w powyższych pracach nie udało się znaleźć skutecznej i bezpiecznej metody leczenia ran przewlekłych. Z uwagi na ten fakt nasza interdyscyplinarna grupa stworzona z polskich naukowców rozpoczęła pracę nad opracowaniem od podstaw i badanie skuteczności innowacyjnego ATMP. Produkt został następnie wykorzystany do badania klinicznego w nowej procedurze chirurgicznej w postaci biologicznego opatrunku obejmującego przeszczep bezkomórkowej ludzkiej skóry, który jest zasiedlony multipotencjalnymi komórkami macierzystymi w celu tymczasowego pokrycia rozległych owrzodzeń z powodu przewlekłych ran. Nazwa BIOOPA jest akronimem od biologiczny opatrunek. Składa się on z bezkomórkowego rusztowania, które tworzone jest z powierzchownych warstw skóry pobranych ze zwłok, poddanych procesowi sterylizacji radiacyjnej. Sterylizacja radiacyjna rusztowań wykonywana jest w specjalnie na te potrzeby skonstruowanym elektronowym akceleratorze liniowym Elektronika 10/10 w INCT, Warszawa z energią wiązki elektronów 10 MeV. Następnie tak powstała bezkomórkowa macierz jest zasiedlona komórkami mezenchymalnymi z galarety Whortona (*Whorhon jelly mesenchymal stem cells,* WJ-MSC). Jest to tkanka łączna, która otacza naczynia pępowinowe. MSC, które się w niej znajdują mają właściwości pluripotencjalne i ze względu na swoje właściwości mają przewagę nad komórkami macierzystymi pozyskiwanymi z tkanek dojrzałych takich jak szpik kostny czy tkanka tłuszczowa [22].

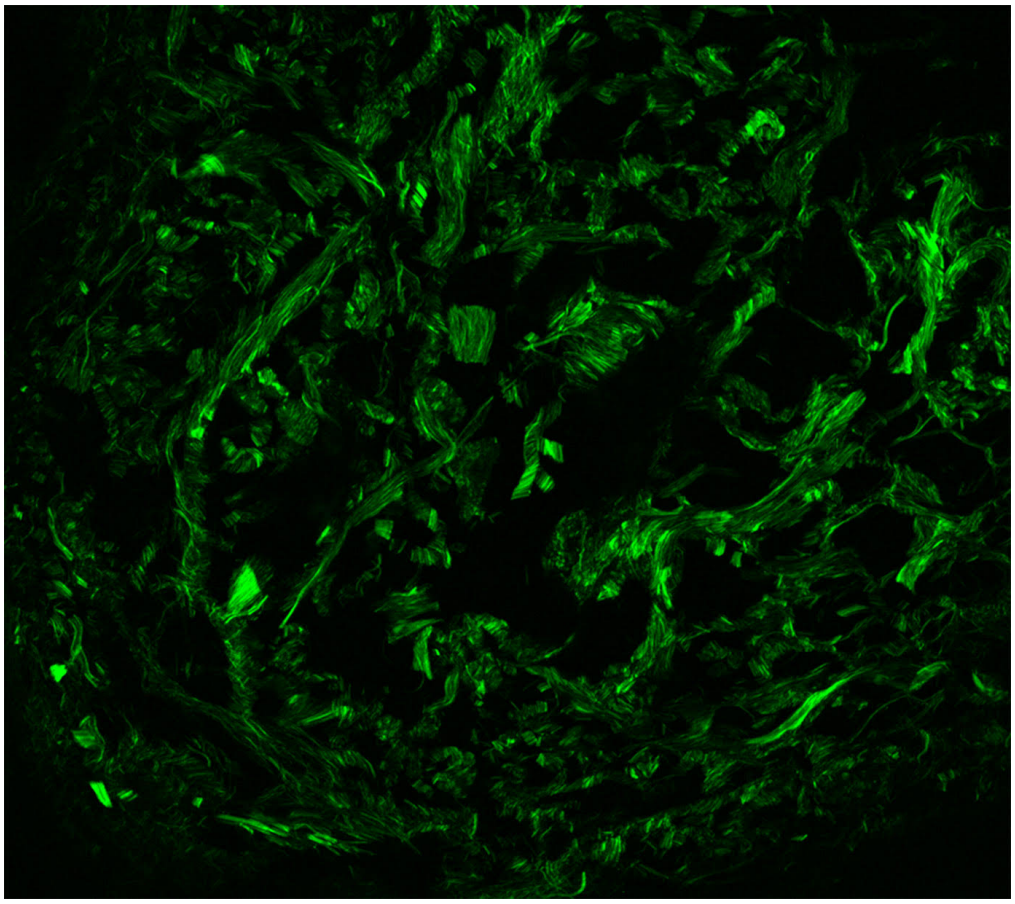
Rozdział II Założenia i cel pracy

Celem prowadzonych badań była ocena bezpieczeństwa i skuteczności polskiego, innowacyjnego opatrunku biologicznego w leczeniu ran przewlekłych/chronicznych w ciężkiej postaci EB. Pierwszy etap badań przedklinicznych i klinicznych w ramach Hospital Exemption został opisany w opublikowanym artykule pt. ***„New Treatment of Wound Healing With Allogenic Acellular Human Skin Graft: Preclinical Assessment and In Vitro Study”*** (tłum. „*Nowa metoda leczenia ran przy użyciu allogenicznego, acelularnego przeszczepu skóry - badanie przedkliniczne i badanie in vitro*”); Transplantation Proceedings, doi:10.1016/j.transproceed.2020.02.115. Założeniem pracy było przeprowadzenie badań mających na celu ocenę bezpieczeństwa i analizę immunogenności stworzonego opatrunku biologicznego w postaci acelularnej skóry

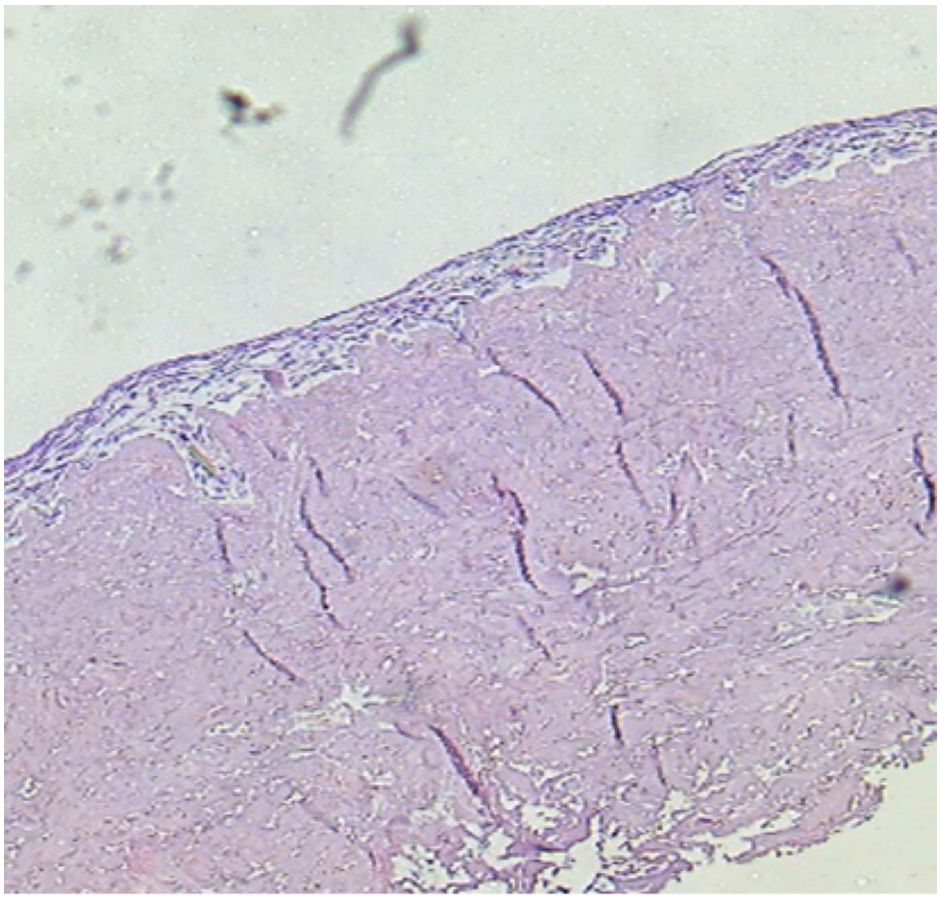
zasiedlonej komórkami macierzystymi. Badanie przedkliniczne i badania *in vitro* zostały zatwierdzone przez komisję bioetyczną (KB/2019 14.01.2019; KB/177/2015). Za pomocą badań immunohistochemicznych, histologicznych, mikroskopii elektronowej i konfokalnej potwierdzono epitelizację i neowaskularyzację opatrunku biologicznego. Ponadto opatrunki charakteryzowały się niską immunogennością, potwierdzoną badaniami histologicznymi i proliferacją limfocytów T *in vitro*. (Ryc. 14).



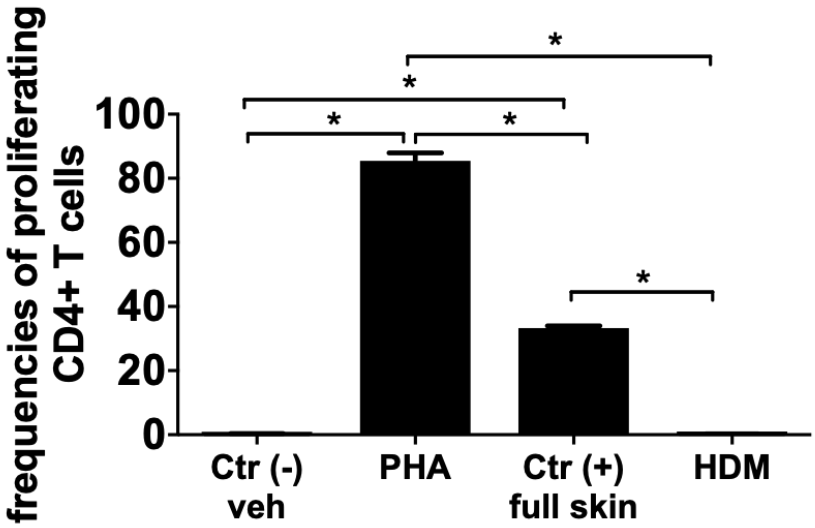
Rycina 1. BIOOPA — produkt leczniczy terapii zaawansowanej, bezkomórkowy odpowiednik ludzkiej skóry sterylizowany promieniami UV zasiedlony komórkami macierzystymi z galarety Whortona.



Rycina 2. Badanie mikroskopem konfokalnym ze skaningiem laserowym przy użyciu techniki generowania drugiej harmonicznej ujawnia strukturę włókienek kolagenowych w bezkomórkowej macierzy skóry po decelularyzacji i promieniowaniu rentgenowskim 35 kG (Bar 1⁄4 50 mm).



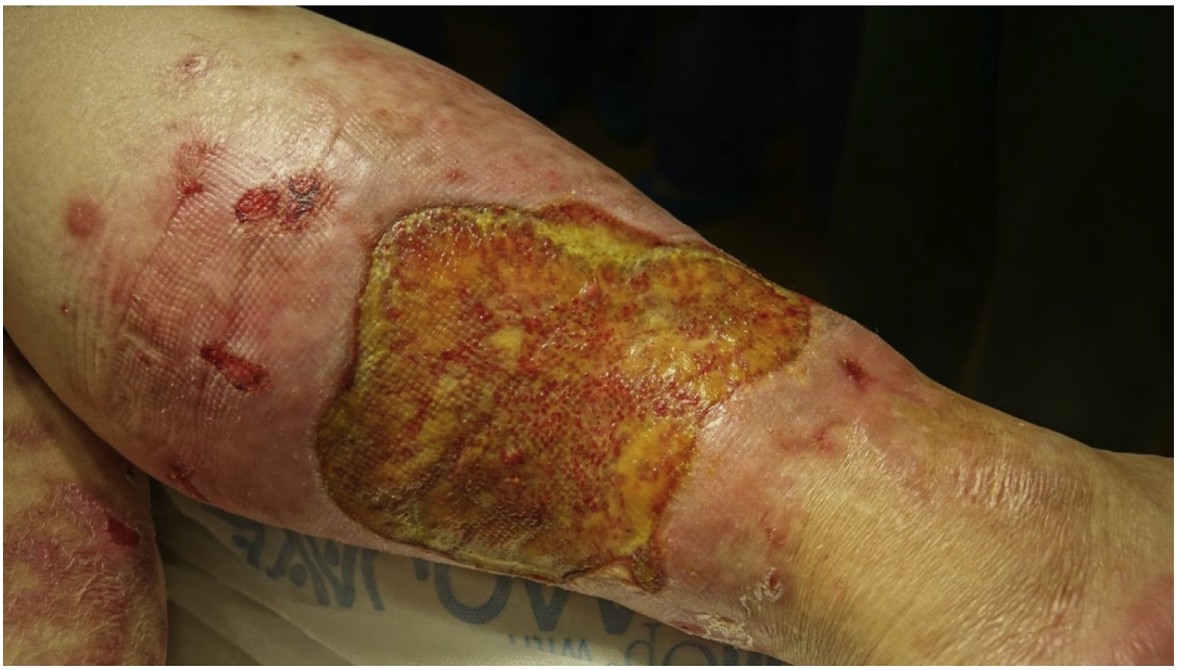
Rycina 3. Barwienie hematoksyliną i eozyną rusztowania zasiedlonego komórkami mezenchymalnymi z galarety Whartona. Po 72 h hodowli mezenchymalne komórki macierzyste tworzą na rusztowaniu wielowarstwową strukturę przypominającą ludzki nabłonek.



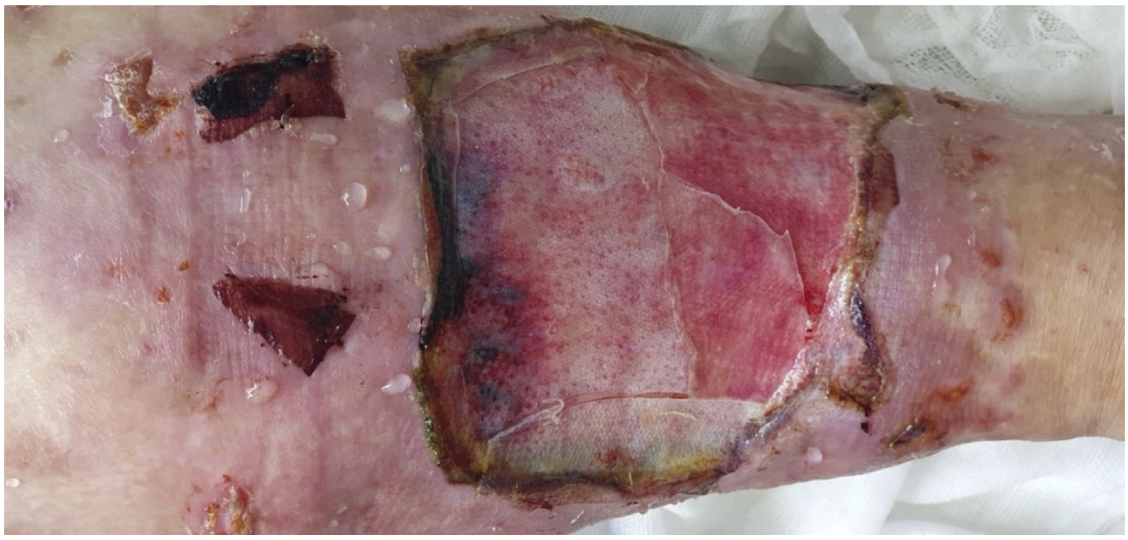
Rycina 4. Częstość proliferacji komórek CD4+T. Ctr (-) veh - kontrola ujemna (nośnik); Ctr (+) - pełna skóra, kontrola pozytywna (normalna pełna skóra); HDM, macierz skórna człowieka; PHA, fitohemaglutynina (proliferacja kontroli mitogennej). *P < 0,05.

Kolejnym etapem badań naukowych/klinicznych było badanie *in vivo*, które polegało na wszczepieniu opatrunku na ranę chroniczną u 50-letniej pacjentki z EB. Zgodnie z protokołem badania klinicznego zaakceptowanego przez Urząd Rejestracji oraz po otrzymaniu pozytywnej opinii Komisji Bioetyczne w celu oceny bezpieczeństwa i skuteczności terapeutycznej w leczeniu ran przewlekłych u chorych z EB. Wyniki badań zostały opublikowane w Transplantation Proceeding pt. ***„Transplantation of a New Biological Product in Rare Diseases, Such as Epidermolysis Bullosa: Response and Clinical Outcome”*** (tłum. „*Przeszczepienie nowego produktu biologicznego w chorobach rzadkich, takich jak Epidermolysis Bullosa: odpowiedź i skuteczność kliniczna*”); Transplantation Proceedings, doi:10.1016/ j.transproceed. 2020.02.119.

Podczas okresu obserwacji stwierdzono gojenie się rany, zmniejszenie dolegliwości bólowych, świądu i poprawę jakości życia pacjentki (Ryc. 5,6). Badania te zostały zrealizowane przez dofinansowanie Narodowego Centrum Badań i Rozwoju w ramach projektu „Praktyki profilaktyczne i leczenie chorób cywilizacyjnych – STRATEGMED” (nr grantu STRATERMED2/269807/14/NCBR/2015).

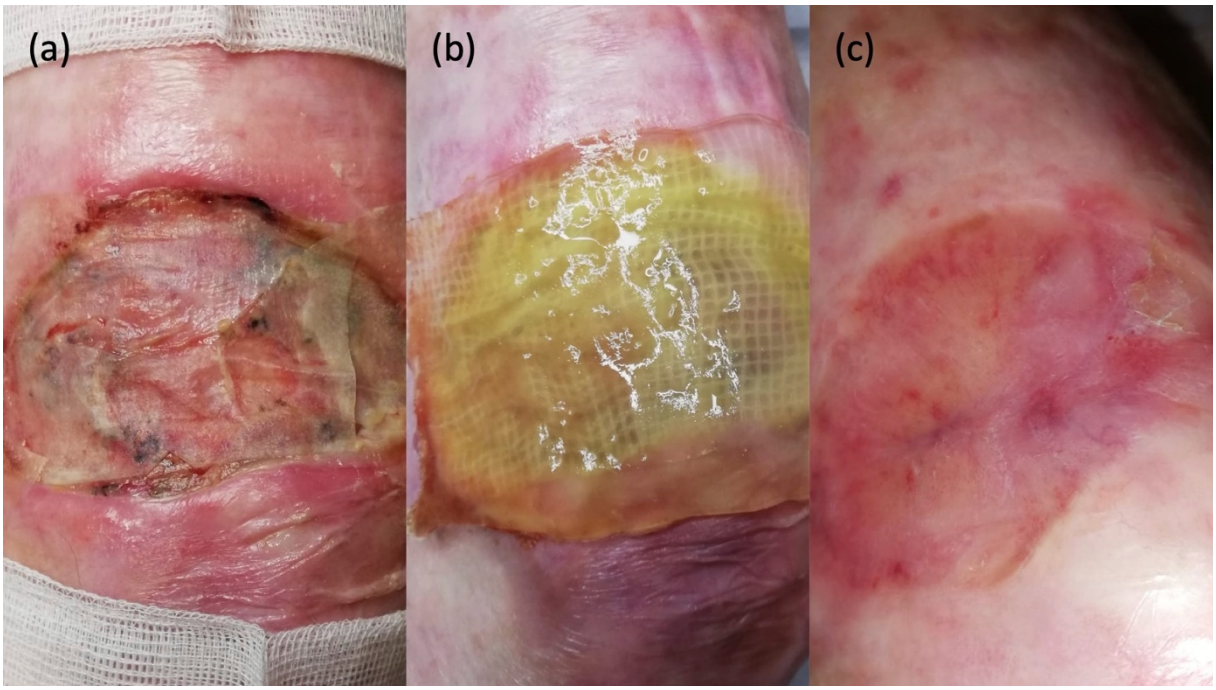


Rycina 5. Dzień 0, zabieg: przewlekła rana w okolicy kolana pokryta przygotowanym acelularnym rusztowaniem u 20-letniej pacjentki z EB (allogeniczny, bezkomórkowy odpowiednik ludzkiej skóry).



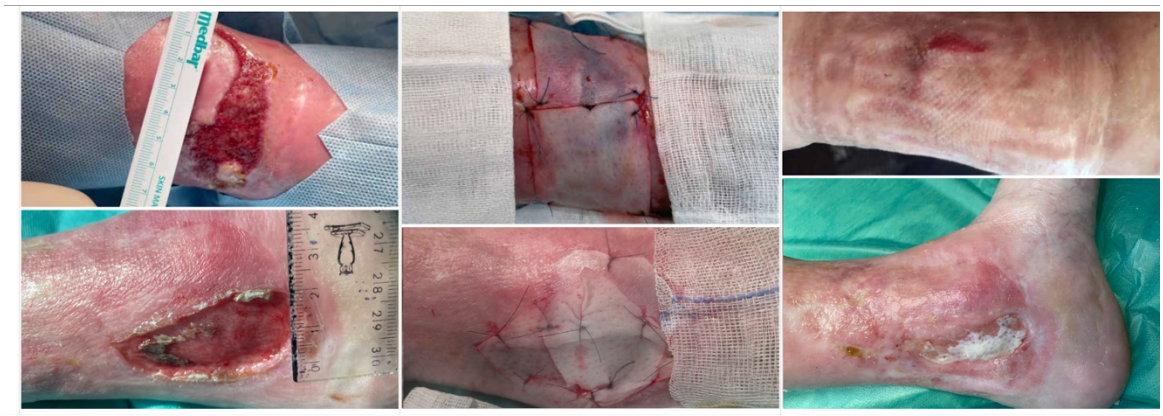
Rycina 6. Obserwacja rany 30 dni po zabiegu.

Przedstawione wczesne wyniki bezpieczeństwa i skuteczności opatrunku biologicznego w leczeniu ran chronicznych stanowiły podstawę do rozszerzenia badań na większej grupie pacjentów (Ryc. 7a-c). W ramach badania klinicznego I/II fazy pt. „*The development of innovative advanced therapy medicinal product (biological dressing of the human race) in the treatment of Epidermolysis Bullosa and other chronic wounds*" (tłum. *Rozwój i badania innowacyjnego opatrunku biologicznego terapii zaawansowanej (opatrunek biologiczny z rasy ludzkiej) w leczeniu Epiermolysis Bullosa i innych ran chronicznych*" Edura CT Number: 2018-003890-91) wykonałam procedury chirurgiczne u dziesięciu pacjentów z przewlekłymi ranami w przebiegu niewydolności żylnej (C-6). Podczas badania procedurze przeszczepienia opatrunku BIOOPA poddano 30-tu pacjentów z ranami chronicznymi w przebiegu EB.



Rycina 7. Opatrunk BIOOPA; a - dzień 0, zabieg: przewlekła rana w okolicy kolana pokryta przygotowanym acelularnym rusztowaniem u 20-letniej pacjentki z EB (allogeniczny, bezkomórkowy odpowiednik ludzkiej skóry); b - rusztowanie opatrumku zasiedlone 30 milionami WJ-MSC w 5 ml 5% roztworu albuminy ludzkiej pokrytej opatrunkiem z chlorheksydyną (Bactigras) i żelem kolagenowym; c - wyniki po 30-dniowej obserwacji tej pacjentki z EB: Wszystkie techniki badania wykazały naciek komórek gospodarza i neowaskularyzację opatrunku biologicznego.

Dodatkowo do badania włączono dziesięciu pacjentów z owrzodzeniami żylnymi. Podczas pięciomiesięcznej obserwacji u badanej grupy pacjentów (40 chorych) stwierdziliśmy zmniejszenie powierzchni ran lub ich całkowite ich wygojenie, ewidentne zmniejszenie redukcję dolegliwości bólowych i zmniejszenie świądu. Wynikiem tego była poprawa jakości życia badanych (Ryc. 8).



Rycina 8. Pacjenci z owrzodzeniami żylnymi przed procedurą, doba 0 - założenie opatrunku BIOOPA, wyniki po 30-dniowej obserwacji.

Badania kliniczne potwierdziły jednoznacznie bezpieczeństwo i skuteczność opatrunku BIOOPA. Obserwacja kliniczna w długoterminowym okresie oraz wyniki w grupie chorych z ranami różnego pochodzenia potwierdzają ważne znaczenie metody w leczeniu ran przewlekłych różnego pochodzenia. Dotychczasowe wyniki potwierdziły, że konieczna jest rejestracja i zatwierdzenie produktu leczniczego ATMP w postaci sterylizowanych radiacyjnie allogenicznych przeszczepów skóry bezkomórkowej stosowanych jako opatrunek biologiczny do leczenia chronicznych owrzodzeń i ran w chorobach rzadkich w celach terapeutycznych i poprawy jakości życia.

Wyniki badania klinicznego potwierdziły założenia i cel pracy i obejmowały ocenę bezpieczeństwa oraz skuteczność opatrunku biologicznego w postaci acelularnej, autogenicznej skóry sterylizowanej radiacyjnie zasiedlonej komórkami macierzystymi z galarety Whortona w leczeniu ran przewlekłych.

Rozdział III Kopie opublikowanych prac

Review of the Latest Methods of Epidermolysis Bullosa and Other Chronic Wounds Treatment Including BIOOPA Dressing" (tłum. „*Przegląd najnowszych metod leczenia Epidermolysis Bullosa i innych ran przewlekłych włączając opatrunek BIOOPA*") Dermatology and Therapy, doi: 10.1007/s13555-021-00578-w.

Dermatol Ther (Heidelb)
https://doi.org/10.1007/s13555-021-00578-w

REVIEW

Review of the Latest Methods of Epidermolysis Bullosa and Other Chronic Wounds Treatment Including BIOOPA Dressing

Magdalena Nita · Jacek Pliszczyński · Maciej Kosieradzki · Piotr Fiedor

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ABSTRACT

Epidermolysis bullosa (EB) is a hereditary genetic skin disorder, classified as a type of genodermatosis, which causes severe, chronic skin blisters associated with painful and potentially life-threatening complications. Currently, there is no effective therapy or cure for EB. However, over the past decade, there have been several important advances in treatment methods, which are now approaching clinical application, including gene therapy, protein replacement therapy, cell therapy (allogeneic fibroblasts, mesenchymal stromal cells), bone marrow stem cell transplant, culture/vaccination of revertant mosaic keratinocytes, gene editing/engineering, and the clinical application of inducible pluripotent stem cells. Tissue engineering scientists are developing materials that mimic the structure and natural healing process to promote skin reconstruction in the event of an incurable injury. Although a cure for EB remains elusive, recent data from animal models and preliminary human clinical trials have raised the expectations of patients, clinicians, and researchers, where modifying the disease and improving patients' quality of life are now considered attainable goals. In addition, the lessons learned from the treatment of EB may improve the treatment of other genetic diseases.

Keywords: Biological dressing; Human skin allograft; Allogenic human skin equivalent; Advanced therapy medicinal product; Epidermolysis bullosa; Rare disease

Key Summary Points
Epidermolysis bullosa (EB) is a hereditary genetic skin disorder, classified as a type of genodermatosis
Currently, there is no effective therapy or cure for EB
Methods of EB wound treatment include autogenic skin transplantation, gene engineering and tissue engineered skin substitutes
Recent achievements will lead to the production of skin substitutes displaying the basic qualities of natural skin

M. Nita · J. Pliszczyński · M. Kosieradzki · P. Fiedor (✉)
General and Transplantation Surgery, Medical University of Warsaw, Nowogrodzka Street 59, 02-014 Warsaw, Poland
e-mail: piotrfiedor@wp.pl

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INTRODUCTION

Epidermolysis bullosa (EB) is a group of autosomal dominant and recessive disorders where injury leads to blistering and skin erosion [1, 2]. Several different subtypes have been described, and the underlying molecular pathology involves mutations in at least ten different genes encoding structural proteins within the dermoepidermal junctions (DEJ) or primary epidermal keratinocytes. One of the most severe clinical forms of EB is recessive dystrophic EB (RDEB). This condition is characterised by widespread fragility of the skin and mucous membranes [3].

In general, wounds and blisters are followed by scarring and an increased incidence of squamous cell carcinoma, which represents the main cause of death in young adults with RDEB [4]. Affected individuals also suffer from many non-skin-related complications, including chronic anaemia, osteopenia and tactile hallucination [5]. RDEB is caused by loss-of-function mutations in the gene encoding type VII collagen, COL7A1 [6].

Currently, there is no causal cure for EB. Phenytoin, psoralen plus UVA photochemotherapy, tetracycline, systemic glucocorticoids, and antimalarial drugs are not very effective, and EB therapy is mainly focused on local wound healing and avoiding injury. Surgical treatment consists of skin transplant, repairing mitten hand deformities, and splinting and dealing with visceral complications (e.g., jejunostomy tubes, oesophageal dilation). Other important complementary therapies include physiotherapy, genetic counselling, aggressive infection treatment, nutritional supplementation, and regular monitoring for malignant skin tumours. Skin and wound care in EB is specific to both the type of EB and the individual wounds of each child. The availability of dressings and personal preference of the patient are also important when choosing materials. Although an ideal dressing for EB is yet to be developed, many suitable dressings are currently available. It is difficult for wounds to heal, and chronic wounds are common. Factors that adversely affect healing include anaemia, malnutrition, infection, and itching. Parallel advances in gene and stem cell therapies are approaching combinatorial therapies that promise clinically significant and lifelong improvement [7–9]. Recent studies using hematopoietic stem cells, mesenchymal stromal cells, or stem cells in the treatment of EB have demonstrated the potential to treat severe cases permanently and effectively. In addition, advances in the use of gene therapy and gene editing techniques, combined with the development of induced pluripotent stem cells from patients with EB, allow for autologous therapies derived from a renewable patient-specific cell population [10, 11].

The low success rate of conventional wound management methods necessitates the production of skin substitutes, such as a layer of keratinocytes inoculated on a biocompatible carrier. This creates a microenvironment suitable for both fibroblasts and epithelial cells, which can assist in repairing the wound and reducing the undesirable results of the abovementioned methods. The multidisciplinary field of tissue engineering was created through the collaboration of biomedical and biomaterial engineers, cell and molecule scientists and clinicians with the aim to develop viable, advanced medical devices to restore the normal functions of damaged tissue. Thanks to this interdisciplinary field, many bioengineered skin substitutes have been developed as an appropriate dressing over the damaged area to treat healing-resistant wounds, which can be as effective or even surpass conventional wound-healing methods [8, 12].

In this article, we describe recent methods of treating genodermatoses, using EB as an example, and present a discussion of their advantages and limitations as effective therapies. Thsi study was approved by an ethics committee (KB / 2019 14/01/2019; KB / 177/2015). This article is based on previously conducted studies and does not contain any new studies with human participants or animals performed by any of the authors.

METHODS OF WOUND TREATMENT

Dressings

Recently developed innovative dressing materials include bioelectric dressings, double-layered silk gelatine and dressings with new ointments such as Triterpine. An "ideal" burn wound dressing was described as having non-adhesive, absorbent properties and antimicrobial activity. Goertz et al. [13] described a solidifying gel that dissolves in a specific temperature range, providing an interface that is better accepted by patients with superficial wounds. Their new gel is liquid at room temperature and hardens to a gel consistency at normal body temperature or above, which causes less pain and leads to better results regarding staining, leakage, and odour compared to silver sulfadiazine gauze. Another promising dressing recently described in non-human studies involves a gelling dendrite dressing based on hydrogel, with three-stage bonds that can dissolve on demand. The possibility of applying the gel, which solidifies in a few minutes, greatly simplifies the process of applying the dressing. In vivo studies have shown that these gels ensure effective haemostasis and prevent infection while providing a moist wound-healing environment. An important feature of this dressing is the ability of clinicians to dissolve the dressing on demand for atraumatic removal [7]. Antibacterial gel dressings based on chitosan (Opticell Ag^{+}) have recently been introduced, which provide a moist, adaptable, highly absorbable antimicrobial dressing to reduce dressing changes and alleviate pain. Catrix powder (bovine cartilage powder; Cranage Healthcare International) is a medically recommended alternative, and early studies suggest faster healing of blisters after Catrix application [14]. Honey, in the form of impregnated dressings and ointments, is effective in both the treatment of chronic wounds and reducing the biological load [15]. Cutimed Sorbact dressings remove bacteria through hydrophobic interactions. They are coated with a fatty acid derivative that attracts bacteria to the dressing, where they are bound [16]. Preliminary studies have shown that this dressing is effective for wound healing in people with chronic EB-related wounds. Dressings containing polyhexanide, such as Suprasorb X1 PHMB (Activa Healthcare, Lohmann & Rauscher, UK), provide antimicrobial treatment for critically colonised and infected wounds, and they are recommended for long-term application [13]. The polymer membrane dressing (PolyMem, Ferris, OH, USA) contains a cleaning agent (surfactant), which reduces the biological load and allows the healing of resistant wounds. Polymeric membrane dressings have the advantage of being "self-contained" without the need for a non-adherent primary or secondary dressing to protect or manage exudation. The frequency of dressing changes depends on personal choice, available time, and level of exudation [17]. Infected or critically colonised wounds require more frequent dressing changes. The use of honey products and polymeric dressings on the membrane initially increases the amount of exudate, so before starting, the patient must commit to daily dressing changes.

Ibuprofen-soaked (Biatain-Ibu) dressings have proven to be helpful for some wounds; however, they are not licensed for children aged under 12–15 years [18].

Autogenic Skin Transplantation

Skin transplantation is an old technique that was rediscovered during World War I and II, becoming the main way to heal wounds. Padget and Hood invented the dermatome, an indispensable device still used to this day to collect large portions of skin. In 1929, Brown developed a split-thickness skin transplantation technique, distinguishing between full-thickness, medium-thickness, and epidermal transplants [19].

Skin grafts can be categorised by graft thickness, geometry, and source. Depending on the thickness of the graft, a distinction is made between split-thickness skin grafts (SSG) and full-thickness skin grafts (FTSG) [20].

Split-thickness skin grafts consist of epidermis and some layers of dermis. Different types

of SSGs can be identified: thin SSG (0.15–0.3 mm), medium SSG (0.3–0.45 mm), and thick SSG (0.45–0.6 mm) [21].

FTSG consist of epidermis, dermis, and various layers of subcutaneous tissue. The amount of dermis plays a key role in determining the mechanical, functional, aesthetic, and transplant trophic properties. In fact, a thicker transplant has better mechanical, functional, and aesthetic properties, but neovascularisation and revascularisation occur with some difficulties and last for at least 5 days [21, 22].

Split-thickness skin grafts are characterised by a poor cosmetic outcome. In addition, SSGs contain fewer tissues requiring revascularisation after implantation; therefore, thin grafts can be used to treat wounds with reduced blood supply [21].

The method employed for supplying and covering skin defects (FTSG or SSG) varies depending on the centre and the experience of the surgeon. However, there is little evidence in the literature of the superiority of one method over the other, and long-term results may vary slightly. There are several factors to consider: availability of donor sites and their potential to heal, delaying the onset of contraction, the likelihood of a successful transplant, and patient selection [21].

It has been suggested that FTSG may delay contract recurrences better than SSG. However, the use of FTSG is often less successful than applying SSG, leading to potential scar formation. In addition, the site of skin collection shows much poorer healing in patients with EB, limiting the skin surface that can act as a source and increasing the likelihood of scar contracture at the site of collection [23].

Problems can be minimised by only collecting the epithelium as a "split" graft. With this technique, healing is faster, and the epithelium can be collected from any place where there are no damaged skin and blisters with purulent substance. Recurrent contracture is more common within the first 6 months, but healing at the donor site is more predictable and usually occurs within 2 weeks. The authors have used this technique several times [22].

Gene Engineering

Until recently, EB treatment only consisted of symptomatic treatment. With advances in the field of genetics, new and exciting therapies are being proposed to address the cause of skin fragility in these patients, including replacement of the abnormal protein (e.g., collagen VII in RDEB) and bone marrow transplantation.

Recent studies have suggested that the delivery of allogeneic fibroblasts to the skin of patients with RDEB may be beneficial for improving skin adhesion and increasing the deposition of type VII collagen at the dermoepidermal junction. There is promising data in patients with RDEB treated with immune myeloablative chemotherapy and allogeneic stem cell transplantation, which resulted in better wound healing, reduced blistering, and increased collagen VII deposition at the dermoepidermal junction. Viral vectors are the most common form of gene therapy for the treatment of genetic disorders. Retroviral, lentiviral, and adenoviral vectors have been developed for RDEB gene therapy. One study used a retroviral vector for the transduction of fibroblasts, which were then evaluated and injected into a mouse model of RDEB. Transduced fibroblasts have been shown to express functional C7, embed it as mature anchor fibrils, and ensure improvement based on both in vitro and in vivo evaluation. The first application of gene therapy in RDEB patients was a retroviral vector used for the transduction of keratinocytes containing full-length human COL7A1. Transduced keratinocytes were then cultured in a good manufacturing practice device to generate corrected epidermal sheets for autologous therapy. These external autologous transplants were tolerated for 12 months with positive results. Adenoviral vectors have been similarly used to correct RDEB cells with both fibroblasts and keratinocytes and then to determine the induced pluripotent stem cell (iPSC) line for future therapeutic applications. These improved iPSCs were then differentiated into keratinocytes that were able to express C7 and transform into layers both in vitro and in vivo. Lentiviral vectors have also been developed for C7 gene therapy. Recently, a

lentiviral vector containing the codon-optimised COL7A1 gene was developed and used to correct RDEB fibroblasts. Corrected fibroblasts have been shown to express full-length functional C7 in vitro and embed C7 in DEJ in skin grafts in immunodeficient mice. These approaches may be useful to develop the combinatorial therapies needed to address the systemic problems of this disease [24–28].

Although encouraging, more research is needed to determine the long-term safety and effectiveness of this modality. Until then, the goals of treatment are to optimise wound healing and minimise disability caused by blisters [29, 30].

Tissue Engineered Skin Substitutes

Tissue engineering is rapidly progressing from basic research to commercial applications.

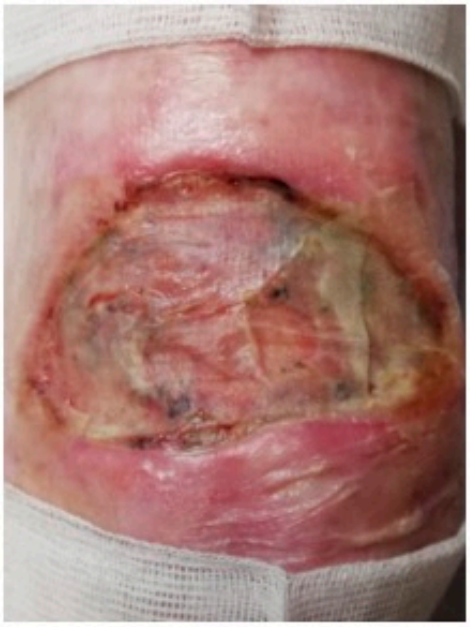

Fig. 1 Day 0, procedure: wound covered with the prepared graft (allogenic human skin equivalent)

Many skin substitutes have been produced by in vitro methods. They are available in various forms, mainly classified into epidermal, dermal, and dermoepidermal or composite skin analogues, which may consist of cell-based or cell-free scaffolds [31].

Biocompatibility, biodegradability, non-carcinogenic cross-linking, cost-effectiveness, risk of infectious diseases, and prevention of immune system stimulation are all factors that need to be considered to create safe and high-quality engineering requirements for the skin. The main approach in the engineering of skin substitutes is the culture of primary skin cells, such as stem cells, fibroblasts, keratinocytes, melanocytes, and Langerhans cells, in a natural or biosynthetic scaffold mimicking the three-dimensional (3D) structure of normal cells [32].

Although there is a wide range of tissue engineering products available on the market, almost none of them meet all the requirements for real skin, including deep skin processes, appropriate vascularisation, and normal pigmentation [32].

The first product to apply tissue engineering to EB is the autologous cultured epidermal substitute (CES). Pioneering work by Green [33] demonstrated that it is possible to grow epidermal keratinocytes as layered sheets from a single cell suspension, and multilayer sheets obtained in this way are very effective for healing burns and wounds in patients with EB.

Along with the acceptable demand for skin components, several types of two-layer skin substitutes consisting of both epidermal and dermal components have been developed. Bell et al. [34] developed a cultured skin substitute (CSS), the equivalent of live skin, which consists of a collagen gel with fibroblasts covered with keratinocytes. Boyce [35] developed a CSS consisting of collagen/glycosaminoglycan with fibroblasts deposited by keratinocytes. Kuroyanagi et al. [36] also developed a cultured skin substitute consisting of a spongy collagen matrix with fibroblasts applied over keratinocytes. These two-layer skin substitutes are designed to be a permanent cover for FTSGs [31, 37, 38]. Recent tissue engineered skin substitutes are included in Table 1.

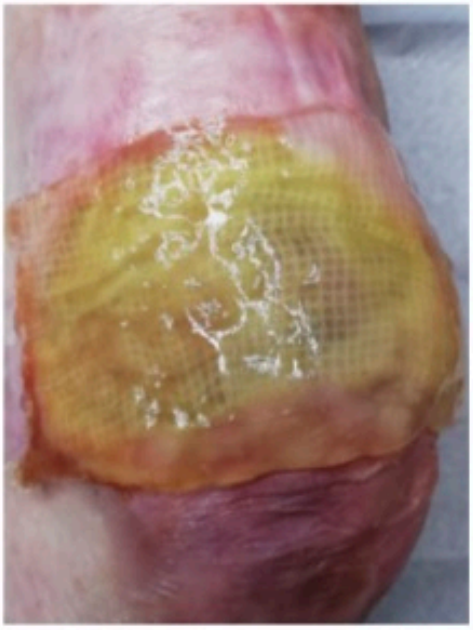

Fig. 2 BIOOPA dressing: an acellular human skin allograft seeded with multipotent stem cells

DISCUSSION

Despite tremendous progress in understanding the molecular genetics and the underlying pathological mechanisms of EB over the past few decades, there is still no cure. There have been many preclinical attempts to develop new treatments for EB. The goal of these therapeutic approaches was to correct the primary genetic defect at the DNA, mRNA, or protein level using induced pluripotent stem cells (iPSC) or keratinocyte-based gene correction, the use of protein therapies for antisense oligonucleotides, and the use of medications that trigger premature termination codon reading. Other potential treatment strategies include disease-modifying therapies that relieve the symptoms and deal with the inflammatory and fibrotic processes responsible for specific EB phenotypes. Although such reports are promising, any potentially effective EB therapies are currently at preclinical stage and are not yet available on the market. Thus, the search for new methods of treatment is still of great importance. Blistering and wound formation along with accompanying pain and discomfort is part of life of patients with EB. Pain is difficult to manage; patients very often reported reluctance to use painkillers because of side effects. Patients with EB are also sensitive to heat because of the inability of skin to sweat effectively. Another difficulty is the application and change of dressing. This process depends on size and positioning of wounds and may be time consuming. Patients reported that preparation and application of dressing may take from 30 min to 7 h. Patients also reported dressing adhesion to fragile skin, even those designed to be low adherent. Tissue engineered skin substitutes seem to be the perfect agent for treatment of wounds of patients with CF and other chronic wounds [43]. The complex EB phenotype triggers a cascade of secondary pathological consequences; therefore, successful treatment will likely require a combinatorial strategy. Although the use of HCT to treat EB appears promising, it is a procedure with inherent risk, including transplant failure, graft versus host disease, a transiently compromised immune system, and side effects resulting from the chemotherapy regimen. Although the use of HCT for EB treatment carries an inherent risk and not all treated patients show significant improvement, the potential for HCT or other stem cell therapies is promising and should be continued and improved. Studying the biological mechanisms of stem cell therapies such as HCT and gene therapy will be valuable in guiding our future approaches. The subset or subsets of cells from an HCT transplant that are effective in producing C7 and mediating wound healing have not been sufficiently characterised, although some studies have provided insight into which cells may be responsible. Identifying these subgroups may help modify the transplant protocol or improve therapy in a way that promotes greater C7 production in patients who do not respond well to HCT. EB wounds are different in individuals, but there are common problems such as risk of infection,

Table 1 Recent tissue engineered skin substitutes

Type	Description
Apligraf	Two-layer skin substitute composed of dermis and epidermis equivalents. The epidermis and skin layers contain appropriately cultured keratinocytes and fibroblasts obtained from newborn foreskin. Bovine type I collagen is also present in the skin layer, which promotes the growth and differentiation of cells [37]. It has a positive influence on wound healing, providing extracellular matrix components, essential growth factors, and cytokines. A decrease in immune system stimulation in the recipient's body has been reported because Apligraf does not contain antigen-presenting cells, such as macrophages and dendritic cells. There have been no reports of the rejection of bovine collagen or alloantigens expressed on keratinocytes or fibroblasts [39]. Apligraf has a short shelf-life, and its use is associated with high costs. Nevertheless, studies have reported a positive effect of this dressing in EB wound care [40]
Biobrane	Synthetic two-layer skin substitute consisting mainly of type I swine collagen around a 3D nylon filament, with a layer of ultra-thin semi-permeable silicone film as an epidermal layer, which controls the loss of skin fluid [41]
BIOOPA	BIOOPA is an advanced therapy medicinal product, made of an acellular human skin matrix prepared from superficial layers of human skin (10 × 10 cm) harvested from a deceased donor. The acellular dermal matrix is an allograft tissue that is chemically processed to remove all epidermis and dermis cells with molecular and physiological structure of collagen fibers. The structure is sterilized by radiation, and then the matrix is colonized with 30 million mesenchymal stem cells derived from Wharton's jelly in human albumin solution. This skin substitute does not induce patient immune response because of the removal of all cells. Additionally, acellularization reduces the risk of disease transmission [29, 30] (Figs. 1, 2, 3)
OrCelTM	Two-layer composite consisting of a type I bovine collagen matrix, into which cultured neonatal keratinocytes and foreskin fibroblasts are implanted to form the dermis [41]. Its scaffolding is thicker than that of Apligraf, and the patient's cells penetrate the 3D scaffold after transplantation. OrCelTM is used in patients with recessive dystrophic EB (RDEB) [42]. In addition, it stimulates wound healing through cytokines and growth factors such as TGF-α, fibroblast growth factor-1 and keratinocyte-1 growth factor, which are released at the affected site. However, bovine collagen increases the risk of transplant rejection and disease transmission [38]

delicate skin, exudate, and malodour. EB wounds cover large areas of patients' bodies and are referred to as hard-to-heal wounds.

In addition, wound healing is a complex process, and it is unclear whether there are many types of cells, which are responsible for important processes needed for sufficient long-term improvement of EB skin, i.e., wound healing, C7 production, reproduced epithelium, and long DEJ thermal stability. There may certainly be immune cells that are important in the early stages of wound healing and for extracellular matrix production that do not contribute to long-term skin populations. On the contrary, there may be some subsets of stem cells, such as MSCs or blood-derived stem cells, which contribute to the cellular compartments of wounded skin by differentiation or trans-differentiation, but which require specific conditions and time to yield significant therapeutic effects beyond the initial waves of differentiated immune cells. It is necessary to carefully analyse these aspects to understand the complexity associated with using stem cell therapy in the treatment of EB. Additional therapies include antifibrotic or anti-inflammatory drugs, C7 protein therapy, and treatment with methods other than non-stem-cell therapy, such as treatment with genetically modified cells.

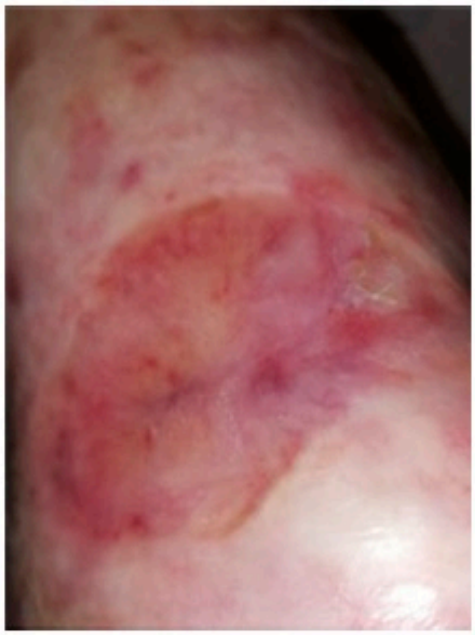

Fig. 3 Result at 30-day follow-up. All examination techniques revealed host-cell infiltration and neovascularisation of the biological dressing. It was characterised by low immunogenicity, as confirmed by histopathology and in vitro T-cell proliferation assays

In conclusion, recent data on animal models and preliminary clinical trials have created significant hope for the development of new and effective EB therapies. Although the promise of a cure is still elusive, several disease-modifying therapies are emerging, and with further refinement and additional clinical testing, translational research in EB is significant and is gradually changing the lives of patients for the better. The lessons learned from EB treatment may have a significant impact on improving the management of other forms of EB and other genetic diseases.

The concept of treating inherited disorders of connective tissue with bone morrow transplant is not new. In fact, the history of EB is somewhat analogous to research conducted approximately 2 decades ago on osteogenesis imperfecta, a genetic disorder that manifests as excessive bone fragility with cracking resulting from a defective type I collagen gene. A series of experiments conducted using the allogeneic bone marrow cells of children with severe osteogenesis defect was carried out following encouraging preclinical trials [44]. Preliminary observations indicated a significant improvement in the mineral content of the body and the microscopic bone structure, which were associated with a reduced frequency of fractures and accelerated growth.

However, the observed clinical improvement was not maintained over time in this group of patients, raising questions about the regenerative capacity of donor-derived mesenchymal progenitor cells, and the lack of persistent donor osteogenesis was considered to reflect an internal program or exogenous signalling environment that suppressed the ability of the transmitted stem cells to differentiate [45].

Based on the latest applications of different types of stem cells (embryonic, prenatal, and adult stem cells), endothelial cells, and melanocytes, combined with significant improvements in the engineering of biocompatible materials such as collagen, HA, elastin, polylactic acid, polylactic-*co*-glycolic acid, and polyethylene glycol, there is now hope for the effective treatment of incurable wounds. Recent achievements will lead to the production of skin substitutes displaying the basic qualities of natural skin, including sweat glands and hair bulbs, as well as even pigmentation and improved healing of scars in the future [31]. However, further research and efforts are crucial for creating truly natural skin-mimicking substitutes. There has been significant progress towards treating patients with EB through different approaches. However, the current approaches are not a cure for this destructive disease, and the risks of some of these procedures should be weighed against their potential benefits. Advanced and innovative strategies with improved safety profiles, which are currently being developed, are clearly required for the successful treatment of this group of currently incurable diseases.

PERSPECTIVES

The future of skin regeneration and wound healing lies in the fields of tissue engineering and regenerative medicine. To obtain an ideal skin substitute, one should consider a variety of features, such as improved vascularity through the application of bioreactors to support vascular formation, longer life, and integration with the tissue of the host. Scaffold polymers, growth factors, and cell lines should ideally mimic the natural structure and function of the skin in the most efficient way. To this end, the addition of melanocytes and hair follicles to scaffolds produced with 3D technology should be considered. Microfluidic dermal printing and automatic tissue paper printing are new techniques that will revolutionise tissue engineering strategies. Skin substitutes are currently attracting a lot of attention, and much experimental research is required to improve the safety and effectiveness of stem cells and engineering materials to meet the demand for high-quality and profitable products manufactured according to standard protocols.

Progress is being made, but there is still much to be done to achieve a cure for EB. Future approaches should be forward thinking. For example, regarding gene therapy, it may be safer and more beneficial in the long term to fix the gene rather than provide an artificial, external source of cells. From a stem cell point of view, the use of stem cells with an internal therapeutic benefit, such as hematopoietic stem cells, may provide a better systemic benefit than treatment with other cellular options [26–28]. While difficult, fixing the genetic component or the cellular component of EB may be the best approach to achieving lasting benefits.

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Compliance with Ethics Guidelines. This study was approved by an ethics committee (KB / 2019 14/01/2019; KB / 177/2015). This article is based on previously conducted studies and does not contain any new studies with human participants or animals performed by any of the authors.

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Chapter

Surgical Treatment of Wounds Using Stem Cells in Epidermolysis Bullosa (EB)

Magdalena Nita, Jacek Pliszczyński, Andrzej Eljaszewicz, Marcin Moniuszko, Tomasz Ołdak, Katarzyna Woźniak, Sławomir Majewski, Cezary Kowalewski, Artur Kamiński, Dariusz Śladowski, Zbigniew Zimek, Maciej Kosieradzki and Piotr Fiedor

Abstract

Epidermolysis bullosa (EB) is a group of hereditary skin diseases, or genodermatoses, characterized by the formation of severe, chronic blisters with painful and life-threatening complications. Despite the previous and ongoing progress in the field, there are still no effective causative treatments for EB. The treatment is limited to relieving symptoms, which—depending on disease severity—may involve skin (blisters, poorly healing wounds caused by the slightest mechanical stimuli, contractures, scarring, pseudosyndactyly) and internal organ abnormalities (esophageal, pyloric, or duodenal atresia; renal failure; and hematopoietic abnormalities). The last decade saw a series of important discoveries that paved the way for new treatment methods, including gene therapy, bone marrow transplantation, cell therapy (allogenic fibroblasts, mesenchymal stem cells [MSCs], and clinical use of induced pluripotent stem cells. Tissue engineering experts are attempting to develop skin-like structures that can facilitate the process of healing to promote skin reconstruction in injuries that are currently incurable. However, this is incredibly challenging, due to the complex structure and the many functions of the skin. Below, we characterize EB and present its potential treatment methods. Despite the cure for EB being still out of reach, recent data from animal models and initial clinical trials in humans have raised patients', clinicians', and researchers' expectations. Consequently, modifying the course of the disease and improving the quality of life have become possible. Moreover, the conclusions drawn based on EB treatment may considerably improve the treatment of other genetic diseases.

Keywords: biological dressing, human skin allograft, allogenic human skin equivalent, Advanced Therapy Medicinal Product, Epidermolysis Bullosa, Rare Diseases

1. Introduction

Epidermolysis Bullosa (EB) is a group of heterogeneous genetic conditions (genodermatoses) characterized by skin fragility and blister formation. These blisters, or bullae, may form spontaneously or as a result of slight mechanical injuries. EB is estimated to occur in 1 person per 50,000 live births.

EB constitutes a group of conditions with diverse clinical courses. Depending on the type of abnormalities in the specific genes, the course, severity, and location of lesions may vary. EB is a result of abnormal connection between the epidermis and dermis. The epidermis, which is the most superficial layer of the skin, constitutes an important barrier between the body and its external environment. The epidermis prevents the loss of water and protects the body against ultraviolet radiation and pathogens. The dermis contains blood vessels, nerve endings, and skin appendages. Under normal conditions, the epidermis and dermis are tightly connected via protein molecules [1–6].

2. The epidermis – structure and functions

The epidermis is the outermost part of the skin and serves as a barrier protecting the body against pathogens, ultraviolet radiation, and excessive loss of water. The epidermal layers, listed from the deepest to the most superficial, include the basal, spinous, granular, and cornified layers. The basal layer is composed of keratinocytes, which undergo intense cell divisions. The newly formed cells differentiate as they progress towards the epidermal surface, eventually becoming dead, anuclear cells (corneocytes) that have no mitochondria. Since they are surrounded by a lipid layer, corneocytes form an impermeable barrier. The epidermis is strongly and permanently connected to the dermis via a cytoskeleton and hemidesmosomes. (**Figure 1**) [7–9].

The course of EB may be severe if the condition is due to a lack of key adhesion proteins, for example as a result of loss-of-function mutations in laminin 332 or collagen VII genes. Conversely, isolated amino acid substitutions typically lead to a mild fragility of the skin. The genetic and allelic heterogeneity of EB is due to

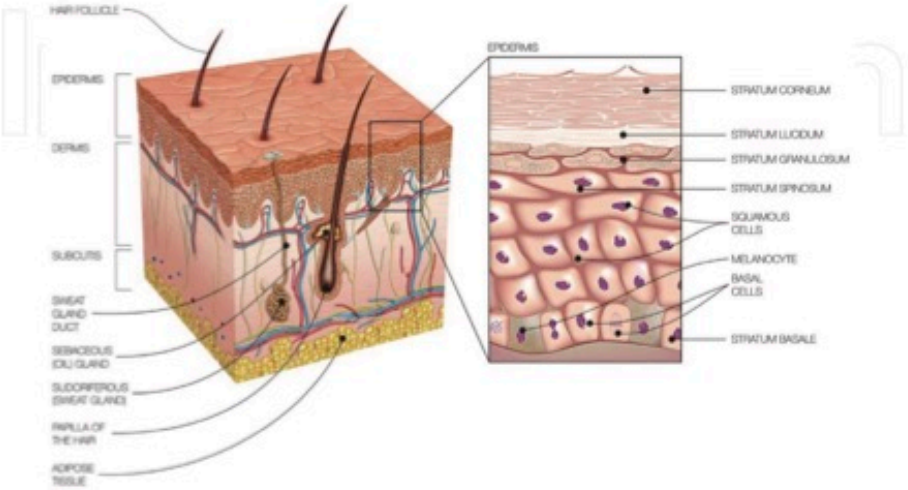

Figure 1.
Skin structure. (from private sources MN).

pathological gene variants in 20 different genes. The genes associated with EB encode intracellular, transmembrane, or extracellular proteins that constitute structural components of the cytoskeleton (keratin 5 and 14), extracellular matrix (integrin α6β4, collagen XVII, laminin 332, collagen VII, α3 integrin, kindlin-1), or intercellular adhesions (desmoplakin, plakophilin, plakoglobin).

3. Epidermolysis Bullosa

The key clinical manifestation of EB is a tendency to develop skin lesions in response to mechanical stimuli, even those of a very low magnitude. The most common lesion types include blisters, milia, pigmented lesions, erosions, epidermal defects, and scars. Other characteristic features of the condition are nail plate changes, ranging from dystrophy to a complete loss. Another common symptom is hair loss and—in severe cases—alopecia. Blisters, erosions, and scars developing near joints may result in contractures and tissue adhesions due to scarring. The lesions that develop on hands and feet (which are most prone to mechanical injuries) may result in pseudosyndactyly. Contractures exacerbate hand and foot deformities, leading to disability ("cocoon hand", or "mitten hand" deformities).

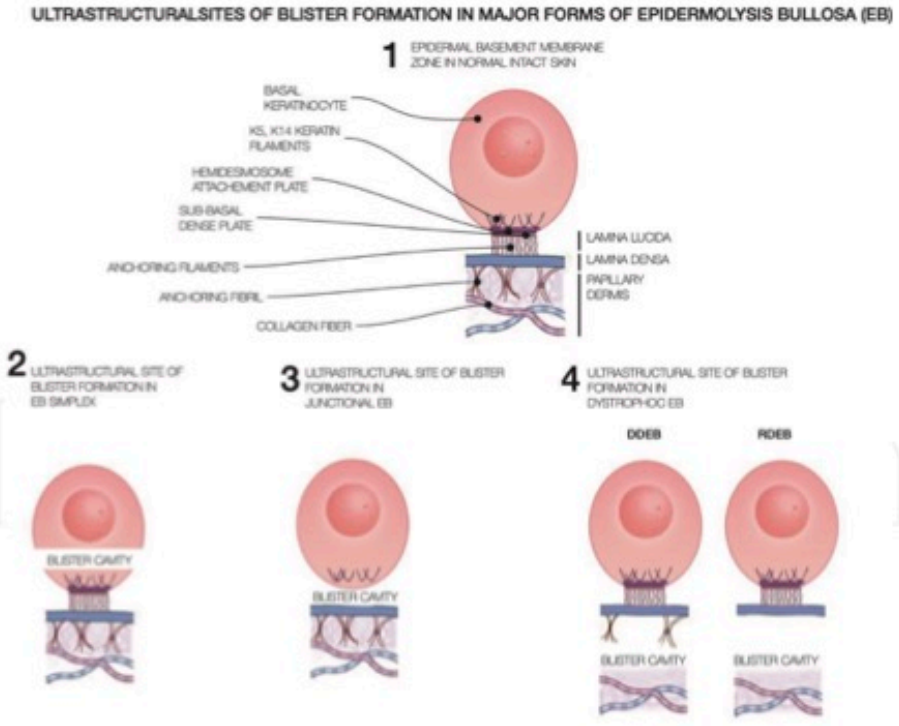

Figure 2.
Ultrastructural sites of blister formation in major forms of epidermolysis bullosa EB. 1. In intact skin, the ultrastructural regions of the epidermal basement membrane zone consist of basal keratinocytes and the hemodesmosomal plaque, the lamina lucida, the lamina densa, the upper papillary dermis 2. in eb simplex (EBS), blisters arise within the lower portion of basal keratinocytes 3. In junctional EB (JEB) blisters form within the lamina lucida 4. In dystrophic EB (DEB), blisters develop below the lamina densa. Anchoring fibris are reduced in number in dominant DEB (DDEB) and absent or rudimentary in recessive DEB (RDEB). KRT5,KRT14 and keratin 5 and keratin14 respectively (s. https://plasticsurgerykey.com/epidermolysis-bullosa/).

Severe forms of EB additionally involve internal anomalies in the oral cavity, esophagus, trachea, lungs, urinary catheter, or urinary bladder. Intestinal tract erosions, ulcerations, and scarring lead to strictures, which may result in difficulty swallowing (dysphagia) and necessitate a feeding jenunostomy to provide enteral nutrition. Oral manifestations of EB may include the tongue adhering to the floor of the mouth (ankyloglossia); a narrowed oral opening (microstomia); and difficulties in chewing and swallowing, which result in malnourishment, osteopenia, osteoporosis, growth retardation, and eating disorders, leading to cachexia. Oral lesions may cause oral hygiene problems, which leads to caries. Perianal erosions and ulcerations cause severe pain during defecation, which contributes to constipation. Possible ocular manifestations involve marginal blepharitis, eyelash loss, ectropion, adhesions between the palpebral and bulbar conjunctivae (symblepharon), and corneal blistering, which may lead to blindness. Other manifestations include treatment-refractory anemia, iron deficiency, and hypoalbuminemia. Due to chronic ulcerations and an impaired protective function of the epidermis, EB patients may develop skin cancer (squamous cell carcinoma [SCC]) in their thirties or forties (**Figure 2**) [10–15].

3.1 Classification

EB is a result of mutations in approximately 20 genes that encode structural and enzymatic proteins responsible for forming and maintaining the connections between the epidermis and dermis. The most common mutations occur in one of three genes: *KRT5*, *KRT14*, or *TGM5*.

- KRT5: The protein encoded by this gene is a member of the keratin gene family. The type II cytokeratins consist of basic or neutral proteins which are arranged in pairs of heterotypic keratin chains coexpressed during differentiation of simple and stratified epithelial tissues. This type II cytokeratin is specifically expressed in the basal layer of the epidermis with family member KRT14. Mutations in these genes have been associated with a complex of diseases termed epidermolysis bullosa simplex. The type II cytokeratins are clustered in a region of chromosome 12q12-q13. (RefSeq, Jul 2008)

- KRT14: This gene product, a type I keratin. At least one pseudogene has been identified at 17p12-p11.

- TGM5: This gene encodes a member of the transglutaminase family. The encoded protein catalyzes formation of protein cross-links between glutamine and lysine residues, often resulting in stabilization of protein assemblies. This reaction is calcium dependent. Mutations in this gene have been associated with acral peeling skin syndrome (RefSeq, Oct 2009). [https://www.genecards.org/]

EB can be classified into three main types, which can be further divided into subtypes. This classification is based on anomalies in various protein molecules and each of the resulting EB types has a different clinical course.

- simple epidermolysis bullosa (SEB) involves epidermal anomalies

- junctional epidermolysis bullosa (JEB) involves basement membrane anomalies

- dystrophic epidermolysis bullosa (DEB) involves anomalies of the dermis

The diagnosis is made based on a thorough microscopic examination of a skin sample. The examination helps determine the exact layer of the skin where tissue separation causes blister formation. There are several layers that can be identified under a microscope in a skin cross-section. If the blisters form within the epidermis, the patient is diagnosed with SEB; if they form within the lamina lucida, the patient is diagnosed with JEB, and if they form just underneath the lamina densa, the patient is diagnosed with DEB (**Table 1**).

Overview of EB classification

Subtype	Phenotype	Inheritance	Gene affected
EB simplex — Intraepidermal			
EB simplex, localized	Palmoplantar blistering from birth or early infancy, with subsequent keratoderma in affected areas	AD	*KRT5 or KRT14*
EB simplex, severe	Early generalized blistering at or soon after birth; congenital areas of denuded skin may be present; can be life threatening in first year of life; classically, tense clustered 'herpetiform' blisters arise with minimal trauma or spontaneously; development of confluent palmoplantar keratoderma; nail dystrophy common	AD	*KRT5 or KRT14*
EB simplex, intermediate	Generalized, although less severe blistering than EB simplex, severe	AD	*KRT5 or KRT14*
EB simplex with mottled pigmentation	Blistering from birth of intermediate severity; additional mottled or reticulate macular pigmentation typically of the neck, upper trunk and acral skin; punctate keratoderma; nail dystrophy may develop	AD	*Predominantly KRT5; less frequently KRT14*
EB simplex, migratory circinate	Vesicles from birth, on a background of inflammatory migratory circinate erythema that fades to leave post-inflammatory hyperpigmentation; nail dystrophy possible	AD	*KRT5*
EB simplex, intermediate with cardiomyopathy	Marked erosions in limbs at birth, healing with dyspigmentation and atrophic burn-like scars; keratoderma, nail-thickening and onychogryphosis possible; diffuse alopecia has occasionally been reported; dilated cardiomyopathy develops later in young adulthood	AD	*KLHL24*
EB simplex, intermediate with PLEC mutations	*Autosomal dominant disease is mild with mainly acral blistering; autosomal recessive has an intermediate presentation*	AD or AR	*PLEC*
EB simplex, intermediate with muscular dystrophy	Generalized blistering with variable-onset myopathy including possible cardiomyopathy; focal plantar	AR	*PLEC*

Overview of EB classification			
Subtype	**Phenotype**	**Inheritance**	**Gene affected**
	keratoderma and nail dystrophy; mucosal involvement is common; upper respiratory tract stenosis has been reported		
EB simplex, severe with pyloric atresia	More severe, widespread generalized blistering or loss of skin at birth with pyloric atresia; early mortality within a few months of birth	AR	*PLEC*
EB simplex, autosomal recessive, KRT5 or KRT14	Generalized blistering, intermediate or severe; keratin 5 abnormalities tend to have a more severe phenotype; absence of keratin 5 associated with widespread skin disease and early mortality; improvement of blistering with age is not expected	AR	*KRT5 or KRT14*
EB simplex, localized or intermediate with BP230 deficiency	Early-onset blistering, relatively mild, usually with acral predominance; plantar keratoderma	AR	*DST*
EB simplex, localized or intermediate with exophilin 5 deficiency	Generalized intermittent blistering and skin fragility; mild mottled pigmentation may be evident	AR	*EXPH5*
EB simplex, localized with nephropathy (CD151 deficiency)	Early blistering, with pretibial predominance; poikiloderma may be seen; early alopecia; extracutaneous involvement manifests as oesophageal webbing and nephropathy	AR	*CD151*
Junctional EB, severe	Blistering may be mild at birth and localized to periungual, buttock and elbow regions; overgranulation develops, particularly on orofacial and periungual regions, with development of bulbous nail folds; alopecia is common; dental enamel defects are usual; a hoarse cry is often a feature; usually fatal within the first 2 years of life	AR	*LAMA3, LAMB3 and LAMC2*
Junctional EB, intermediate	Less severe than above, with a reduced tendency to develop exuberant granulation tissue; elevated risk of SCC in adulthood	AR	*LAMA3, LAMB3, LAMC2 and COL17A*
Junctional EB with pyloric atresia	Extensive areas of skin loss seen at birth with severe cutaneous fragility; early-onset pyloric atresia, a frequent cause of early mortality, within days or weeks of birth; duodenal and anal atresia may also feature; milder non-lethal variants often show genitourinary involvement	AR	*ITGA6 and ITG84*
Junctional EB, localized	Limited cutaneous fragility, often acral; variable nail and dental defects; normal hair	AR	*LAMA3, LAMB3, LAMC2, COL17A1, ITGB4 and ITGA3*

Overview of EB classification			
Subtype	**Phenotype**	**Inheritance**	**Gene affected**
Junctional EB, inversa	Flexural blistering from birth; dental abnormalities and nail loss	AR	*LAMA3, LAMB3 and LAMC2*
Junctional EB, late onset	Onset in childhood, with often acral fragility; skin fragility is progressive and loss of dermatoglyphs may be seen owing to scarring; variable dental enamel and nail defects	AR	*COL17A1*
Junctional EB–laryngo-onycho-cutaneous (LOC) syndrome	Skin fragility from birth with marked exuberant granulation tissue (greater than that in junctional EB, severe), particularly on face and neck; nail dystrophy and loss with granulation tissue of nail beds; laryngeal granulation can lead to respiratory compromise and death; conjunctival and eyelid granulation with consequent symblepharon, scarring and visual loss	AR	*LAMA3*
Junctional EB with interstitial lung disease and nephrotic syndrome	Variable degree of cutaneous involvement; fatality in early childhood is common; nail dystrophy possible; hair loss may occur	AR	*ITGA3*
Dystrophic EB — sublamina densa			
Intermediate DDEB$_1$	Generalized skin fragility, scarring and milia presenting from birth or early infancy, with prominence over acral sites, elbows and knees; involvement of the mucous membranes may lead to microstomia, ankyloglossia and oesophageal stenosis, although less commonly than in severe RDEB	AD	*COL7A1*
Localized DDEB$_1$	Predominantly acral blistering, scarring and milia seen from birth or early infancy; occasional nails-only presentation, with progressive dystrophy and eventual nail loss; rarely, cutaneous features may predominate over pretibial skin alone (and can present as late-onset disease)	AD	*COL7A1*
DDEB, pruriginosa$_1$	Profoundly pruritic linear cords of papules associated with fragility, scarring and milia on the shins, and occasionally progressing to arms; may present in childhood or adulthood; nail dystrophy is usual	AD	*COL7A1*
DDEB, self-improving$_{1,2}$	Blistering evident at or shortly after birth, usually on extremities where there may be aplasia cutis, whilst scarring and milia may occur; spontaneous resolution of cutaneous fragility within the first 2 years of life	AD	*COL7A1*

Overview of EB classification			
Subtype	**Phenotype**	**Inheritance**	**Gene affected**
Intermediate RDEB[3]	Phenotype similar to that of intermediate DDEB, although greater severity with flexion contractures, limited digital fusion and occasional striate keratoderma	AR	*COL7A1*
Severe RDEB[3]	Widespread blistering from birth, with extensive scarring and development of microstomia, ankyloglossia, oesophageal stenosis, flexion contractures of limbs and pseudosyndactyly; nails are often lost early in disease course; high risk of cutaneous SCC arising in EB wounds.	AR	*COL7A1*
RDEB, inversa[3]	Generalized blistering from birth, of intermediate severity; subsequently, fragility tends to be displayed on flexural sites	AR	*COL7A1*
RDEB, localized[3]	Skin fragility and blistering typically at birth or neonatal period, limited to acral sites such as hands and feet, or occasionally only to pretibial skin, where it may manifest as late-onset disease during adulthood; nail dystrophy and loss usual	AR	*COL7A1*
RDEB, pruriginosa[3]	As for DDEB, pruriginosa	AR	*COL7A1*
RDEB, self-improving[3]	As for DDEB, self-improving	AR	*COL7A1*
DEB, severe[4]	Clinically indistinguishable from severe RDEB, with severe mucocutaneous fragility from birth	Dominant and recessive compound heterozygosity	*COL7A1*
Kindler EB — variable and mixed			
None	Generalized blistering and variable photosensitivity from birth or early childhood, with mucosal fragility; blistering gives way to progressive poikiloderma, initially most marked over dorsal hands and neck; confluent palmoplantar keratoderma and adermatoglyphia may occur; gingivitis and dental disease is a feature; oesophageal narrowing and colitis has been reported; mucocutaneous SCC has been reported, with poor prognosis	*AR*	*FERMT1*

AD, autosomal dominant; AR, autosomal recessive; DDEB, dominant dystrophic epidermolysis bullosa; DEB; dystrophic epidermolysis bullosa; EB, epidermolysis bullosa; EM, electron microscopy; ER, endoplasmic reticulum; RDEB, recessive dystrophic epidermolysis bullosa; SCC, squamous cell carcinoma. 1Major type is DDEB. 2Previously known as transient bullous dermolysis of the newborn baby. 3Major type is RDEB. 4Major type is DEB (dominant and recessive compound heterotygosity). Adapted from consensus guidelines3.

Table 1.
Overview of EB classification [16].

3.2 Heredity

SEB primarily shows an autosomal dominant pattern of inheritance, with the most common mutations in genes *KRT5* and *KRT14*. Autosomal recessive inheritance is less common and caused by mutations in genes *KRT14*, *ITGA6*, *ITGB4* (*this genes encodes a member of the integrin alpha chain family of proteins. Integrins are heterodimeric integral membrane proteins composed of an alpha chain and a beta chain that function in cell surface adhesion and signaling. The encoded preproprotein is proteolytically processed to generate light and heavy chains that comprise the alpha 6 subunit. This subunit may associate with a beta 1 or beta 4 subunit to form an integrin that interacts with extracellular matrix proteins including members of the laminin family. The alpha 6 beta 4 integrin may promote tumorigenesis, while the alpha 6 beta 1 integrin may negatively regulate erbB2/HER2 signaling. Alternative splicing results in multiple transcript variants. [provided by RefSeq, Oct 2015]).*

DSP (This gene encodes a protein that anchors intermediate filaments to desmosomal plaques and forms an obligate component of functional desmosomes), or *PKP1 (Plakophilin proteins contain numerous armadillo repeats, localize to cell desmosomes and nuclei, and participate in linking cadherins to intermediate filaments in the cytoskeleton. This protein may be involved in molecular recruitment and stabilization during desmosome formation).* SEB caused by a *PLEC1(Plakins, with their multidomain structure and enormous size, not only play crucial roles in maintaining cell and tissue integrity and orchestrating dynamic changes in cytoarchitecture and cell shape, but also serve as scaffolding platforms for the assembly, positioning, and regulation of signaling complexes (reviewed in PMID: 9701547, 11854008, and 17499243)* mutation may show an autosomal recessive or autosomal dominant pattern of inheritance.

JEB is primarily caused by mutations in genes *LAMB3, LAMC2, LAMA3 (this genes is a laminin that belongs to a family of basement membrane proteins), COL17A1* (This gen encode *collagen XVII is a structural component of hemidesmosomes, multiprotein complexes at the dermal-epidermal basement membrane zone that mediate adhesion of keratinocytes to the underlying membrane), ITGA6* or *ITGB4* and is characterized by autosomal recessive inheritance. A recent study (2009) showed a possible autosomal dominant inheritance pattern in the case of a mutated *COL17A1*.

DEB is caused by mutations in only one gene, *COLA1*. The location and type of mutation determine the inheritance pattern (autosomal recessive or dominant).

3.3 SEB subtypes

- Koebner type: mutated genes for keratin 5 and 4 (*KRT5*, *KRT4*); lesions are often present at birth or in infancy; characteristic features are hyperkeratotic lesions, hemorrhagic bullae, and erosions.

- Dowling-Meara type: mutated *KRT14* and *KRT5* genes, which encode keratin 14 and 5, respectively; autosomal dominant inheritance; lesions are located primarily on the feet, less commonly in other locations; a relatively mild course.

- Weber-Cockayne type: associated with mutated *KRT5* (region 12q13.13) and *KRT14* (region 17q21.2) genes; characterized by a severe course and herpetiform blisters. Poorly healing blisters and erosions lead to scarring and contractures.

- SEB with muscular dystrophy: a mutated plectin-encoding *PLEC1* gene.

3.4 JEB subtypes

- JEB with pyloric atresia: this rare type of EB results from mutated *ITGB4* and *ITGA6* genes that encode α6 β4 integrin. Skin lesions are accompanied by esophageal, pyloric, and/or duodenal atresia. Enamel hypoplasia is common.

- Herlitz type: mutations in the *LAMA3*, *LAMB3*, and *LAMC2* genes, which encode the polypeptide subunits of laminin 5 (α-3, β-3, and γ-2, respectively). Fatal type of EB, characterized by blisters and erosions over the entire body, which causes multiple infections that may lead to sepsis, loss of proteins (malnourishment), scarring, contractures, defects of large areas of skin.

- Non-Herlitz type: mutations in genes *COL17A1*, *LAMB3*, *LAMC2*, or *LAMA3* encoding laminin 5 and collagen XVII.

3.5 DEB subtypes

- Hallopeau-Siemens type: a mutated *COL7A1* gene, which encodes collagen VII. This type of EB is characterized by scarring, erosions, pseudosyndactyly of the hands and feet; nail plate involvement, esophageal atresia, and corneal ulcers are common.

- non-Hallopeau-Siemens type: mutated *COL7A1* gene, encoding collagen VII.

- Cockayne-Toureine type: autosomal dominant inheritance; mutated *COL7A1* (collagen VII); skin lesion on the limbs.

- Pasini type: possible nail plate involvement; oral and mucosal lesions.

3.6 Diagnostic investigations

A primary diagnosis of EB is based on the clinical presentation. The definitive diagnosis is established after skin samples are examined via immunofluorescence antigen mapping and transmission microscopy.

Diagnosis is confirmed via genetic analysis that determines the type of mutation.

3.7 Differential diagnoses

The differential diagnoses should include congenital dermatoses, herpes simplex virus infections, epidermolytic hyperkeratosis with erosions and blisters, staphylococcal scalded skin syndrome, bullous pemphigoid, neonatal pemphigoid, and gestational pemphigoid.

3.8 Treatment

Management is primarily symptomatic. Surgical treatment mainly involves skin grafting. Importantly, the use of autologous skin grafts is ineffective due to poor healing and chronic wound formation at the donor sites. Plastic surgery procedures play an important role in repairing contractures and pseudosyndactyly of the hands and feet. In the case of esophageal, pyloric or duodenal atresia, various surgical procedures are used to overcome the effects of gastrointestinal strictures (e.g. feeding jejunostomy, endoscopic balloon dilatation).

EB management involves primarily local care of chronic wounds, ulcers, erosions, and blisters. Treatment challenges involve frequent bacterial infections, due to their chronic character, and factors that inhibit healing, such as malnutrition, anemia, itching, or repetitive wound irritation with regular dressing changes, all of which disturb epithelialization. Moreover, wounds may cause severe pain, exacerbated by regular, frequent dressing changes. Importantly, the condition requires life-long care, with the cost of monthly treatment often exceeding several hundred dollars. Therefore, the process of selecting the optimal dressing should include the following parameters: the price, availability, effectiveness, and safety. Other important complementary treatments include physiotherapy, genetic counselling, aggressive treatment of infections, nutritional supplementation, and skin cancer monitoring [17–31].

Despite the enormous advances in our understanding of molecular genetics and EB physiopathology that have taken place over the last several decades, a definitive cure is yet to be discovered. There are many ongoing studies aiming to develop an effective treatment. These studies focus on several potential lines of treatment, including disease modifying treatments to diminish disease severity. Gene therapies, bone marrow transplants, and tissue engineering are receiving the most attention.

Advanced therapy medicinal products (ATMPs) are medicines for human use that are based on genes, tissues or cells. They offer groundbreaking new opportunities for the treatment of disease and injury. ATMPs can be classified into three main types: gene therapy medicines, somatic-cell therapy medicines, tissue-engineered medicines. In addition, some ATMPs may contain one or more medical devices as an integral part of the medicine, which are referred to as combined ATMPs. An example of this is cells embedded in a biodegradable matrix or scaffold.

Gene therapy involves cultures of keratinocytes (obtained from patients with recessive DEB [RDEB]) that have been transduced with a retroviral vector containing full-length cDNA of the *COL7A1* gene (for collagen VII). These cultures are, subsequently, placed onto the patient's wounds in the form of epidermal grafts [32]. Treatment efficacy and collagen VII expression were demonstrated; however, the response lasted up to 12 months. Nonetheless this therapy is safe. One disadvantage of this method is the fact that it can be used in limited areas (at chronic wound sites). This method has been also used in a patient with JEB, in whom the placement of genetically corrected keratinocytes onto chronic wound sites led to successful wound healing. Based on the available reports, gene therapies are promising treatment modalities with a potential therapeutic effect in genodermatoses.

Bone marrow transplant (BMT) and allogenic stem cell transplantation (ASCT) are other very promising treatment strategies. In 2010, Wagner et al. performed ASCT in children with RDEB. Although the patients were not completely cured, their skin blisters were reduced, and skin regeneration was accelerated. BMT in RDEB patients has been reported to improve the clinical status, despite the lack of collagen VII growth in the skin. BMT is an experimental therapy, which is used as part of clinical studies, and currently is not an approved treatment. The risk of death and the uncertain degree and mechanism of the clinical response should be viewed in light of the results of the most recent translational research in RDEB, which reports ASCT to be currently the only therapeutic approach that shows systemic effects in what essentially is a systemic disease. There is a clear need for reports presenting data from extensive clinical studies to establish guidelines and warnings for the use of ASCT in EB treatment.

As pluripotent cells, MSCs have a potential to differentiate into many different types of skin cells, including keratinocytes, endothelial cells, and monocytes. Due to

their immunomodulatory and anti-inflammatory effects, MSCs may play a significant role in wound healing and tissue regeneration. Moreover, MSCs do not trigger an immune response in the recipient, hence there is no need to match the donor's and recipient's human leukocyte antigen (HLA) types [33]. Due to their multidirectional differentiation potential, MSCs have been shown to regenerate collagen VII, which has a beneficial effect on the healing of wounds (including chronic wounds) and improves skin stability. These effects were observed with intradermal administration, which—apart from presenting fewer challenges—does not require as many MSCs as intravenous administration. Most studies have focused on bone marrow-derived MSCs (BM-MSCs). However, their harvesting from the bone marrow is a relatively invasive procedure. Moreover, the multipotent differentiation potential of BM-MSCs diminishes with age. Therefore, MSCs are currently obtained from alternative sources, such as the umbilical cord [34], which can provide up to a billion cells in 30 days, obtained non-invasively. The umbilical cord consists of umbilical vessels surrounded by a connective tissue, referred to as Wharton jelly (WJ). WJ-derived MSCs have a higher proliferative potential and are more homogeneous than those derived from the bone marrow. WJ-MSCs are similar to BM-MSCs in their fibroblast-like phenotype, non-hematopoietic surface markers [35], low immunogenicity [36], multipotent plasticity, and the expression of CD90, CD73, CD105 markers [37]. Moreover, WJ-MSCs seem to have more pronounced pro-angiogenic properties than BM-MSCs; they promote neovascularization and perfusion by releasing paracrine factors and by playing the role of perivascular precursor cells [38]. WJ-MSCs are a highly efficient source of young, non-carcinogenic, and non-immunomodulatory cells [39]. All these properties and the fact that WJ-MSCs are easily available make these cells a promising strategy for treating wounds in EB patients.

Sebastiano et al. propose an innovating cell therapy for RDEB treatment, by developing a state of the art protocol of genetically repaired induced pluripotent stem cells (iPSCs) as to generate sheets of normal skin tissue to treat affected skin areas [40]. Moreover, as numerous stem cells are needed in order to cover the affected surface area, authors outline the necessity for creating personalized iPSCs banks as to provide a constant long-term iPSCs source. Generally, human iPSCs can be generated by reprogramming differentiated somatic cells into pluripotent embryonic stem cells (ESCs) capable of differentiating into ectoderm, mesoderm or endoderm cells. Reprogramming involves the introduction of a known set of genes into the somatic cells, using integrating viral and non-integrating non-viral methods. Following successful reprogramming, somatic cells will express genes and surface proteins similar to ESCs in vitro and will be able to differentiate into any of the three embryonic germ layers.

Tissue engineering: Not unlike patients with extensive burns, patients with EB do not qualify for autologous skin grafts. One solution available to these patients involves the use of allogeneic grafts, which serve to temporarily cover the wound (after 7 days the graft is rejected by the recipient; [41]). Therefore, tissue engineering seems to be a promising solution, as it helps create biopolymer scaffolds to cover the wounds. The idea is to create skin substitutes, which can then be seeded with keratinocytes, fibroblasts, or stem cells. Such polymer materials constitute a microenvironment and provide adequate scaffolds for cell colonization and epithelial cell migration during wound epithelialization. The multi-disciplinary nature of tissue engineering has helped develop many bioengineered skin substitutes, with potential applications as a suitable dressing for treating refractory wounds, such as those in EB patients. The field of tissue engineering has been rapidly transferring from the realm of basic research to commercial applications. There are many in

vitro-generated skin substitutes. They are available in various forms, which include epidermal, dermal, and dermo-epidermal analogs or complex skin analogs, and can be composed of cellular or acellular scaffolds [42–51].

High-quality, safe skin analogs should be cost-effective, biocompatible, biodegradable, and noncarcinogenic, carry no risk of infectious disease transfer, and provoke no activation of the recipient's immune system. Despite a whole spectrum of bioengineered products currently available on the market, there are scarcely any that meet all the requirements of natural skin. Natural skin is composed of the epidermis, dermis, and subcutaneous tissue. It contains appendages, such as sweat glands, nails, and hair, as well as nerve endings and blood vessels. Additionally, natural skin protects the body against the external environment via its thermoregulatory function and its role in maintaining water–electrolyte balance. It also facilitates the perception of pain, heat, and touch; manufactures vitamin D; and shields the body against ultraviolet radiation by the means of melanin-producing melanocytes responsible for skin pigmentation [52, 53]. Due to the wide range of functions performed by human skin, creating its analog is a challenge for tissue engineers.

The first product that has transferred the potential of bioengineering into real-life EB applications is an autologous cultured epidermal substitute (CES). The pioneering study by Rheinwald and Green demonstrated that epidermal keratinocytes from a single-cell suspension can be cultured in the form of sheets, and the resulting multi-layered sheets have proven to be very effective in the treatment of burns and wounds in EB patients. There are many commercially available skin substitutes composed of both epidermal and dermal components. Bell et al. developed a cultured skin substitute (CSS) (an equivalent of living skin) composed of keratinocytes and fibroblasts in a collagen gel. Boyce and Hansbrough developed a CSS composed of a collagen-glycosaminoglycan composite scaffold populated with keratinocytes and fibroblasts. Kuroyanagi et al. developed another CSS, composed of a spongy collagen matrix with keratinocytes and fibroblasts. Such two-layered CSSs are intended to permanently cover full-thickness skin defects. There have been studies on wound healing in EB with the use of OrCelTM, Biobrane, and Apligraf dressings. OrCelTM is a bilayer dressing composed of a bovine-collagen I matrix populated with neonatal foreskin keratinocytes and fibroblasts [54–56]. Despite the fact that OrCelTM exhibits beneficial wound healing properties in RDEB patients—via cytokines and growth factors, such as tumor growth factor alpha (TGFα), fibroblast growth factor 1 (FGF-1), and keratinocyte growth factor 1 (KGF-1)—its bovine collagen component increases the risk of graft rejection and transfer of diseases to the donor [57]. Another bilayer skin substitute is Biobrane, which is composed of a 3D nylon fiber scaffold and an ultrathin semipermeable epidermis-mimicking silicone layer that controls fluid loss [56–59]. The nylon fibers are surrounded by porcine collagen type 1. Jutkiewicz and Noszczyk [60] were the first to report the use of Biobrane in the postoperative hand care in a group of RDEB patients. Apligraf is another bilayer skin substitute composed of dermal and epidermal analogs. The epidermal and dermal layers contain cultured keratinocytes and neonatal foreskin fibroblasts. The dermal layer additionally contains bovine collagen type 1, which facilitates cell growth and differentiation. Apligraf has a short life span, and its use is associated with high costs [57]. Nonetheless, this dressing was reported to be effective in treating EB wounds [61, 62].

Safe and Effective Therapy in the Light of Clinical Trials - New Approach to Treatment by Innovative Method (BIOOPA-ATMP) grant no. STRATERMED2/269807/14/NCBR/2015.

Alternative promising product for the treatment of chronic wounds that occur in EB and other genodermatoses, as well as in burns, is an allogeneic, acellular human skin equivalent sterilized with radiation, and seeded with Wharton's jelly-derived mesenchymal stem cells- WJ-MSCs about the acronym in polish BIOOPA (biological dressing) is an advanced therapy medicinal product composed of a decellularized matrix of the superficial layers of cadaveric human skin (10 cm × 10 cm). Acellular dermal matrix (ADM) is a Chemically/enzymatically processed allograft. This processing removes all epidermal and dermal cells while preserving the molecular and physiological structure of collagen fibers. The scaffold is sterilized via radiation and then seeded with 30 million WJ-MSCs. As a result of decellularization, this skin substitute does not induce an immune response in the recipient and poses a lower risk of transmitting any diseases. In order to assess the safety and efficacy of the BIOOPA dressing, the relevant study was conducted in two stages. During the first stage, in vitro experiments showed BIOOPA viability. All examination techniques demonstrated graft infiltration by host cells and neovascularization of the biological dressing. Moreover, BIOOPA is characterized by low immunogenicity, which was confirmed in histopathology examinations and in vitro T-cell proliferation tests. The second stage of the study was conducted in a group of qualified volunteers with EB and approved by an ethics committee. The 6-month follow-up indicates the safety and efficacy of the BIOOPA dressing, with no infections or necrosis at the graft implantation site observed over the follow-up period. The subjects reported decreased pain and improved quality of life **Figures 3–8** [63, 64].

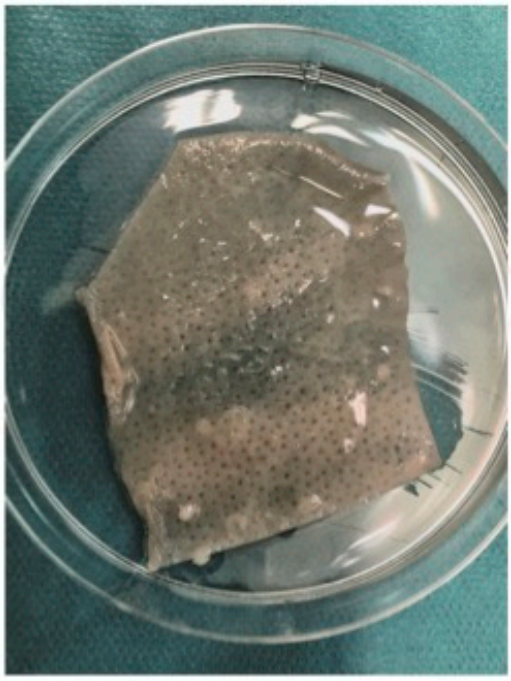

Figure 3.
BIOOPA- Advanced Therapy Medicinal Product (ATMP) acellular human skin equivalent sterilized with ultraviolet radiation.

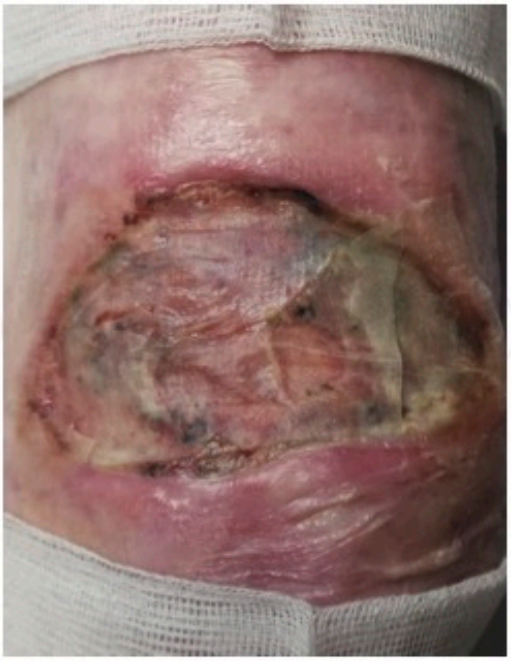

Figure 4.
Day 0, procedure: chronic wound in the knee area covered with prepared graft in the 20-years old patient with EB (allogenic,acellular, human skin equivalent).

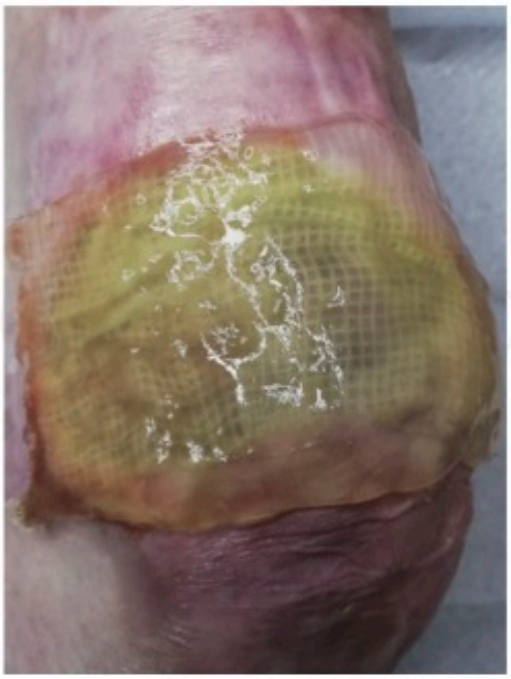

Figure 5.
Bioopa dressing: The scaffold is seeded with 30 million WJ-MSCs in 5 mL of a 5% human albumin solution covered with chlorhexidine-impregnated dressings and collagen gel. The same 20-years old patient with EB (chronic wound in the knee area).

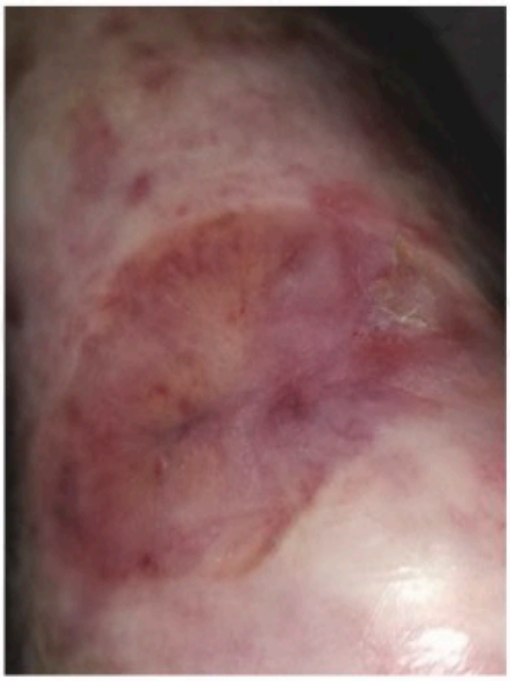

Figure 6.
Results after 30-day follow-up in this patient with EB: All examination techniques revealed host-cell infiltration and neovascularization of the biological dressing. They are characterized by low immunogenicity, as confirmed by histopathology and in vitro T-cell proliferation assays.

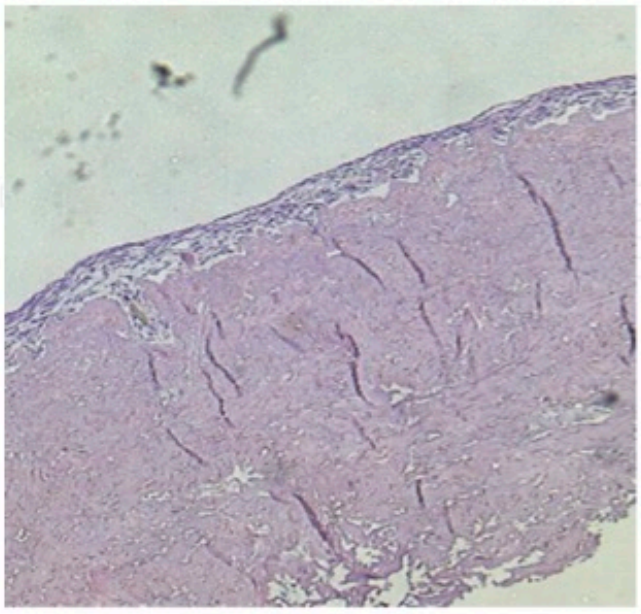

Figure 7.
Hematoxylin and eosin stain of scaffold populated with mesenchymal cells from Wharton's jelly. After 72 hours of culture mesenchymal stem cells create a multilayer structure on the scaffold resembling human epithelium.

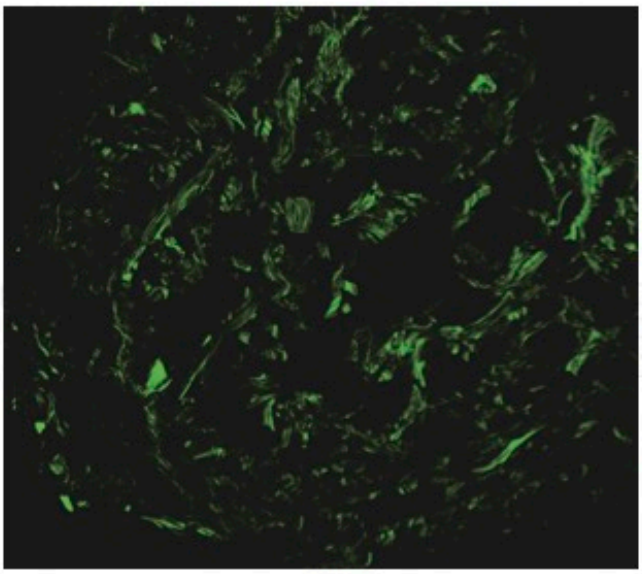

Figure 8.
Laser scanning confocal microscopic study using second-harmonic generation technique reveals the structure of collagen fibrils in acellular dermal matrix after decellularization and X-ray radiation 35 kG (Bar 1/4 50 mm).

4. Conclusion

To date, there is no causative treatment of EB, despite multiple ongoing studies involving gene therapy and bone marrow transplantation. The standard of EB management still involves symptomatic conservative treatment. There is immense hope in therapies with the use of stem cells of various origins (bone marrow, umbilical cord, etc.). Advanced applications of various types of cells (embryonic, prenatal, and adult stem cells, endothelial cells, and melanocytes) and the rapid development of biomedical engineering, which contributes to refining biocompatible materials, such as collagen, hyaluronic acid, elastin, polylactic acid (PLA), poly lactic-co-glycolic acid (PLGA), and polyethylene glycol (PEG), bring hope of effective treatment for chronic wounds of various origin. The most recent developments allow for the manufacture of progressively better skin substitutes, which in the future may exhibit the fundamental characteristics of natural human skin (including sweat glands and hair follicles), more homogeneous pigmentation, and allow for the healing of scars [65]. Thus, further studies and efforts are crucial for creating skin substitutes truly mimicking natural skin. Despite the enormous progress in the treatment of EB, the current treatments are clearly not a definitive cure for this debilitating disease, and the risk associated with some of these procedures must be weighed against their potential benefits. Effective treatment of this, currently incurable, group of diseases requires advanced and innovative strategies with an improved safety profile, such as the ones that are currently being developed [66–77].

The BiOOPA dressing is easily available, safe, and relatively inexpensive, all of which make it a promising therapy for EB-associated wounds. Preliminary results of the BIOOPA study indicate the dressing to be safe and effective to improve the quality of life in study subjects. Currently BIOOPA is evaluated as part of a phase

I/II clinical study during the second year of observation. Our preliminary results of clinical trial strongly suggest, that our innovative dressing is a promising strategy and a tool for clinicians in the search for new opportunities of treatment for this rare condition.

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Author details

Magdalena Nita[1], Jacek Pliszczyński[1], Andrzej Eljaszewicz[2], Marcin Moniuszko[2,3], Tomasz Ołdak[4], Katarzyna Woźniak[5], Sławomir Majewski[6], Cezary Kowalewski[5], Artur Kamiński[7], Dariusz Śladowski[7], Zbigniew Zimek[8], Maciej Kosieradzki[1] and Piotr Fiedor[1*]

1 Department of General and Transplantation Surgery at Medical University of Warsaw, Infant Jesus Clinical Hospital, Warsaw, Poland

2 Department of Regenerative Medicine and Immune Regulation at Medical University of Białystok, Białystok, Poland

3 Department of Allergology and Internal Medicine at Medical University of Białystok, Białystok, Poland

4 PBKM, Polish Stem Cells Bank, Warsaw, Poland

5 Department of Dermatology and Immunodermatology at Medical University of Warsaw, Warsaw, Poland

6 Department of Dermatology and Venereology at Medical University of Warsaw, Warsaw, Poland

7 Department of Transplantology and Central Tissue Bank at Medical University of Warsaw, Warsaw, Poland

8 Institute of Nuclear Chemistry and Technology, Warsaw, Poland

*Address all correspondence to: piotrfiedor@wp.pl

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*„**New Treatment of Wound Healing With Allogenic Acellular Human Skin Graft: Preclinical Assessment and In Vitro Study**”* (tłum. *„Nowa metoda leczenia ran przy użyciu allogenicznego, acelularnego przeszczepu skóry - badanie przedkliniczne i badanie in vitro*”); Transplantation Proceedings, doi:10.1016/j.transproceed.2020.02.115.

New Treatment of Wound Healing With Allogenic Acellular Human Skin Graft: Preclinical Assessment and In Vitro Study

M. Nita[a], J. Pliszczyński[a], C. Kowalewski[b], K. Woźniak[b], A. Eljaszewicz[c], M. Moniuszko[c,d], A. Kamiński[e], D. Śladowski[e], Z. Zimek[f], S. Majewski[b], M. Kosieradzki[a], and P. Fiedor[a,*]

[a]Department of General and Transplantation Surgery, Medical University of Warsaw, Warsaw, Poland; [b]Department of Dermatology, Medical University of Warsaw, Warsaw, Poland; [c]Department of Regenerative Medicine and Immune Regulation, Medical University of Białystok, Białystok, Poland; [d]Department of Allergology and Internal Medicine, Medical University of Białystok, Białystok, Poland; [e]Department of Transplantology and Central Tissue Bank, Medical University of Warsaw, Warsaw, Poland; and [f]Institute of Nuclear Chemistry and Technology, Warsaw, Poland

ABSTRACT

Background. Nonhealing wounds can be a major clinical problem. Impaired wound healing is often related to massive tissue injury, concomitant wound healing deficiencies (chronic wounds), burn injury, or congenital conditions. We propose a novel biological dressing as an alternative surgical approach. The dressing is a form of an allogenic human skin graft equivalent with further use of allogeneic stem cells classified as an advanced therapy medicinal product. This new allogenic acellular human skin graft has been specifically developed to address the clinical indications for dressing wound lesions and promoting tissue repair in specific rare genetic diseases.

Methods. This case report illustrates the use of an acellular human skin allograft seeded with multipotent stem cells in the treatment of tissue injuries (burns), congenital conditions, and chronic wounds. Donor-tissue processing yields an acellular dermal matrix with integral collagen bundling and organization, as well as an intact basement membrane complex.

Results. Preclinical observations show prolonged viability of acellular human skin grafts with multipotent stem cells. This was confirmed with histological and electron-microscopic evaluation of biopsies, which demonstrated host-cell infiltration and neovascularization of the biological dressing. Moreover, the dressings were characterized by low immunogenicity, as confirmed by histology exam and T-cell proliferation assays in vitro.

Conclusion. Our data confirmed the safety and efficacy of the evaluated acellular human skin grafts, which may be used in patients with rare diseases, such as epidermolysis bullosa, burn injuries, and chronic wounds.

CHRONIC wounds have become an increasing medical and economic problem in aging societies, not only in Europe but also in other parts of the world [1–4]. Such wounds can be caused by both local and systemic factors. The former include localized ischemia, necrosis, foreign bodies, abnormal extracellular matrix remodeling, fibroblast dysfunction, infection, lack of response to growth factors, inflammation, edema, and ionizing radiation [5]. Systemic causative factors of chronic wounds include poor nutrition, anemia, autoimmune disorders, peripheral vascular conditions, diabetes mellitus, immunosuppressive and anti-inflammatory agents, and rare genetic diseases [6]. The clinical presentation varies and is largely dependent on two factors: appropriate wound management and the patient's general condition [7,8]. Despite the extensive range of available controversial treatment methods, including wound

*Address correspondence to Prof. P. Fiedor, Medical University of Warsaw, Centralny Szpital Kliniczny WUM, Klinika Chirurgii Ogólnej i Transplantacyjnej, Nowogrodzka 59, 02-014 Warszawa, Poland. Tel: 0048225021734. E-mail: piotrfiedor@wp.pl

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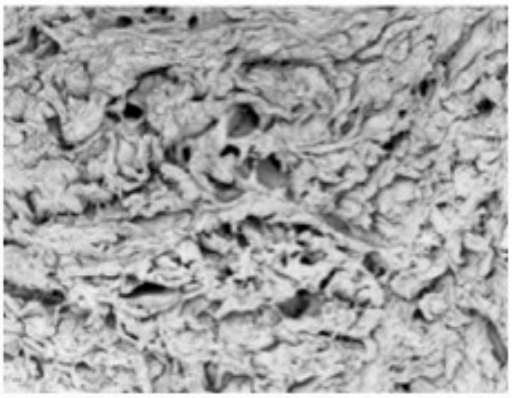

Fig 1. Images of the structure of the skin after removal of the epithelium and decellularization using scanning electron microscopy): cross-section of the acellular, frozen skin. Bar = 500 μm.

debridement, advanced wound dressing, hyperbaric oxygen therapy, compression therapy, and reconstructive surgery [9–12], no optimal supportive treatment for chronic wounds has been found. Thus, clinicians face the considerable challenge of developing a new supportive treatment to optimize the conventional wound management techniques. There is an urgent need for simple and noninvasive, yet effective, means of promoting wound healing.

The aim of our study was to assess the safety and biological effects of a new wound dressing. Our multidisciplinary team began working on the development of an innovative advanced therapy medicinal product in the form of a biological dressing, specifically an acellular human skin graft, which will be seeded with multipotent cells and used to temporarily cover extensive chronic ulcerations.

Preclinical assessments and in vitro studies of this novel dressing indicated that it may be a good alternative surgical approach to chronic wound management.

METHODS

Preparation of Human Cadaveric Skin Graft as an Acellular Human Equivalent

Acellular human skin matrices were prepared from superficial layers of human skin (10 × 10 cm) harvested from a deceased donor.

Sterilization of Allogenic Scaffold Graft

The scaffolds were sterilized with radiation (10-MeV electron beam) generated by an Elektronika 10/10 linear electron accelerator at the Institute of Nuclear Chemistry and Technology, Warsaw [13]. Scaffold samples were covered air-tight with a double layer of flat envelopes made of polymer film. Subsequently, the scaffolds were placed in a single layer in an aluminum box covered with a low-density polyethylene film. Radiation sterilization was conducted at low temperatures ensured by dry-ice pellets, which had been distributed in thin layers underneath and above the scaffold samples. Dry ice (solid carbon dioxide) was used as a cooling agent, as it sublimates under atmospheric pressure at a temperature of −78°C, leaving no residue. Irradiation was conducted by scanning the samples with pulsed electron beams (pulse duration 5.6 μs; pulse repetition frequency 340 Hz; scan width 58 cm; scanning frequency 5 Hz). A 35 ± 0.53-kGy dose was delivered, with the beam current set at 510 ± 0.18 mA, with conveyor speed of 0.42 ± 0.50 m/min. Electron beam energy was measured periodically with an aluminum wedge and a B3 radiochromic film dosimeter, as well as continuously, with a secondary-electron monitor. The dose delivered during the sterilization process was measured periodically with a RISO polystyrene calorimeter [14], and continuously with a beam-current monitor and conveyor speed sensors. B3 film dosimeters had been calibrated against an alanine dosimeter from the National Physical Laboratory, Teddington, Middlesex, UK [15]. The used B3 radiochromic films were processed with a RISO flatbed scanner.

In Vitro Study: Immunogenicity and Quality

This has been confirmed via immunohistochemical, histologic, electron, and confocal microscopy examinations of biopsy samples (Figs 1–3)

Advanced Therapy Medicinal Product

Acellular human skin matrices will be seeded with stem cells commercially available in the Polish Stem Cell Bank.

This study, involving an experimental treatment method with a novel therapeutic product in the form of a human reticular acellular dermis matrix biological dressing, had been approved by the institutional review board and ethics committee. This study is the first step in assessing the safety and efficacy of biological dressings in the form of decellularized human dermis matrix.

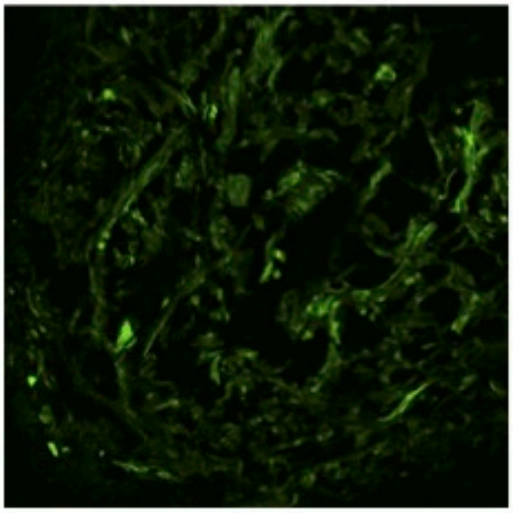

Fig 2. Laser scanning confocal microscopic study using second-harmonic generation technique reveals the structure of collagen fibrils in acellular dermal matrix after decellularization and X-ray radiation 35 kG (Bar = 50 μm).

ETHICS

This study was conducted in compliance with the Helsinki Congress (as revised in 2013) and the Istanbul Declaration. The authors declare no conflicts of interest.

RESULTS

In vitro studies show viability of acellular human skin grafts with multipotent stem cells. All examination techniques revealed host-cell infiltration and neovascularization of the biological dressing. Moreover, these newly designed dressings are characterized by low immunogenicity, as confirmed by histopathology and in vitro T-cell proliferation assays (Fig 4).

DISCUSSION

Chronic wounds have a considerable, adverse impact on patients' quality of life and national health care funds [16]. Such wounds are a common complication of peripheral vascular disease. Current Central European data show that chronic leg ulcers are most commonly due to chronic venous insufficiency, which is the main cause of the lesions in 47.6% of patients with chronic leg ulcers. A total of 17.6% of chronic ulcerations are caused by arterial and venous disorders, with atherosclerosis accounting for 14.5%. Less common causes of chronic ulcerations include vasculitis (5.1%), external factors (3.8%), pyoderma gangrenosum (3.0%), infections (1.4%), neoplasms (1.1%), and calcium (1.1%) [1]. Moreover, there are also other, very rare causes of ulcerations, such as epidermolysis bullosa (reported mostly in younger patients) [17,18].

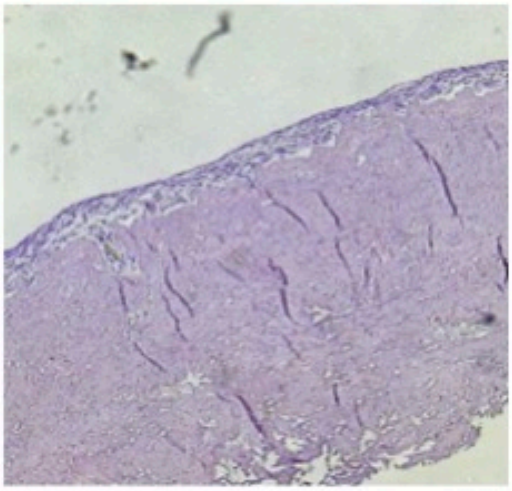

Fig 3. Hematoxylin and eosin stain of scaffold populated with mesenchymal cells from Wharton's jelly. After 72 hours of culture mesenchymal stem cells create a multilayer structure on the scaffold resembling human epithelium.

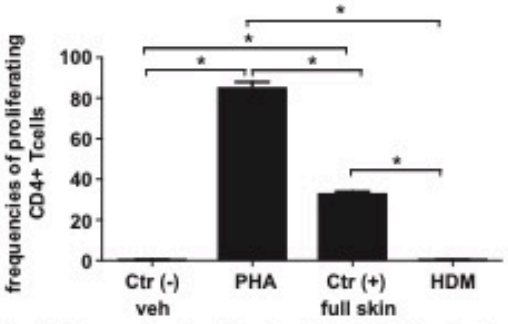

Fig 4. Frequencies of proliferating CD4+T cells. Ctr (-) veh, negative control (vehicle); Ctr (+) full skin, positive control (normal full skin); HDM, human dermal matrix; PHA, phytohemagglutinin (mitogenic control proliferation). *$P < .05$.

One convenient treatment option would be topical treatment, which is easy to apply and is characterized by low toxicity. There are many topical products being currently evaluated as part of proof-of-concept studies or early clinical trials (eg, betulin-rich triterpene extract from birch bark [Oleogel-S10]) [19].

An important factor that impedes wound healing is biofilm formation. Despite several available methods of biofilm management, such as physical removal and the use of systemic or topical antimicrobial agents, there is a need for novel methods, particularly utilizing controlled release of antimicrobial agents [7].

Autologous and allogenic grafts have been routinely used for wound repair. However, autologous grafts are not feasible in case of extensive burns or genetic conditions [20,21]. Tissue engineering involving a combined use of scaffolds, growth factors for promoting wound healing, cells, and gene therapy might ensure the necessary conditions for successful chronic wound healing [22–37]. Yet, the scope of the relevant studies is mostly limited to in vitro assays. Therefore, more in vivo studies are needed before such biological dressings can be used in clinical practice.

CONCLUSIONS

Our in vitro studies indicate that safety and efficacy of the dressing in the form of an allogenic human skin equivalent may have potential clinical utility and potential clinical application using stem cells to treat chronic ulcerations of various etiology and burn wounds, may be indicated for epidermolysis bullosa wounds.

ACKNOWLEDGMENT

The study has been approved by an ethics committee (KB/2019 14.01.2019; KB/177/2015). This study was financially supported by The National Centre for Research and Development in terms of the project "Prevention Practises and Treatment of Civilizations Diseases-STRATEGMED" (grant no. STRATERMED2/269807/14/NCBR/2015).

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„Transplantation of a New Biological Product in Rare Diseases, Such as Epidermolysis Bullosa: Response and Clinical Outcome” (tłum. „*Przeszczepienie nowego produktu biologicznego w chorobach rzadkich, takich jak Epidermolysis Bullosa: odpowiedź i skuteczność kliniczna*”); Transplantation Proceedings, doi:10.1016/ j.transproceed. 2020.02.119.

Transplantation of a New Biological Product in Rare Diseases, Such as Epidermolysis Bullosa: Response and Clinical Outcome

J. Pliszczyński[a], M. Nita[a], C. Kowalewski[b], K. Woźniak[b], A. Eljaszewicz[c], M. Moniuszko[c,d], A. Kamiński[f], D. Śladowski[f], Z. Zimek[e], S. Majewski[b], M. Kosieradzki[a], and P. Fiedor[a,*]

[a]Department of General and Transplantation Surgery, Medical University of Warsaw, Warsaw, Poland; [b]Department of Dermatology, Medical University of Warsaw, Warsaw Poland; [c]Department of Regenerative Medicine and Immune Regulation, Medical University of Białystok, Białystok, Poland; [d]Department of Allergology and Internal Medicine, Medical University of Białystok, Białystok, Poland; [e]Department of Radiation Chemistry and Technology, Institute of Nuclear Chemistry and Technology, Warsaw, Poland; and [f]Department of Transplantology and Central Tissue Bank, Medical University of Warsaw, Warsaw, Poland

ABSTRACT

Background. Epidermolysis bullosa (EB) is a phenotypically diverse group of hereditary blistering disorders involving mutations in 20 different genes. Those debilitating disorders are currently incurable; however, there are a number of promising preclinical trials, where some treatments already approach the stage of early clinical trial. In this paper we introduce a novel surgical approach to the treatment of EB-induced ulcerations.
The purpose of our study was to evaluate the safety and efficacy of a new biological dressing in the form of an allogenic human skin equivalent graft before using multipotent stem cells, classified as an advanced therapy medicinal product.

Methods. Implanted human acellular dermal matrices were prepared from the superficial layers of donated human skin. Scaffold sterilization was conducted via irradiation with the use of a linear electron accelerator. Following water-knife debridement, wounds were surgically covered with accordingly prepared grafts and dressed in burn-injury fashion. Subsequently, the wounds were monitored for infection and viability.

Results. Our data indicate that grafting as a potential new medicinal product was safe and effective in patients with rare diseases, such as EB, and may be used for stem cells to create new Advanced Therapy Medicinal Products. During a 200-day follow-up, we proved the safety of using human scaffolds (allogeneic graft) by observing no apparent infection or necrosis. Instead, we noted fewer required dressing changes, promoted wound healing, pain reduction, and an overall improvement in the quality of life in patients with EB.

Conclusion. The protocol for grafting allogenic acellular epidermal sheets is the most promising treatment for severely affected skin areas in EB patients to date.

EPIDERMOLYSIS bullosa (EB) is a group of hereditary disorders, characterized by recurring skin blisters due to impaired adhesion between the epidermis and dermis [1,2].

EB can be divided into 3 main types: EB simplex, junctional EB, and dystrophic EB. Each of these types of EB is a result of protein molecule abnormalities. Each of the 3 EB types has a different clinical course, with the symptoms ranging from mild to very severe [3–5].

EB-related lesions have a tendency to turn into chronic wounds, which are often colonized by bacteria. The resulting

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*Address correspondence to Prof. P. Fiedor, Medical University of Warsaw, Centralny Szpital Kliniczny WUM, Klinika Chirurgii Ogólnej i Transplantacyjnej, Nowogrodzka 59, 02-014 Warsaw, Poland. Tel: 0048225021784. E-mail: piotrfiedor@wp.pl

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chronic ulcerations have been reported to be a risk factor for invasive squamous cell carcinoma, which is associated with early mortality [6–8]. Therefore, there is a constant need for effective wound treatments in EB patients.

Currently, there is no universally accepted method of causative treatment that could be used in all EB patients [9]. Nonetheless, there are high hopes for future gene therapies aimed at correcting genetic mutations [10,11]. However, until this goal is grasped, the only available treatments are symptomatic and include daily wound care combined with frequent monitoring visits and biopsies to detect any neoplastic lesions as early as possible. These procedures are invasive, painful, time-consuming, and costly. Hence, there is an ongoing unmet need for safe and effective medical treatment to help improve the patients' quality of life [12].

The aim of this study was to develop an innovative medicinal product in the form of a biological dressing composed of an acellular human skin graft that could be seeded with pluripotent stem cells (advanced therapy medicinal product) and used to temporarily cover extensive ulcerations.

METHODS

We developed a new surgical procedure for applying biological dressings in patients with extensive ulcerations concomitant with an impaired wound-healing mechanism (chronic wounds), burn injuries, or congenital conditions (such as EB).

Our study was divided into 2 stages. The first stage involved preclinical assessment and in vitro studies regarding the safety and efficacy of the biological dressing in the form of an allogenic graft of human skin equivalent. The next stage comprised analyses of the response to treatment and clinical outcome in vivo. The clinical procedure was performed in a 51-year-old woman with dystrophic EB and the study had been approved by the Bioethics Committee at Warsaw Medical University (KB/2019 14.01.2019; KB/177/2015). The evaluated patient had provided her consent to undergo an experimental treatment method involving a new therapeutic product in the form of an HR-ADM biological dressing.

Clinical Procedure

We present the case of 51-year-old female patient, suffering from cutaneous blistering since birth, with normal body development and skin condition significantly improving after puberty. Currently, blistering occurs most often over possible trauma sites on feet, legs, dorsal hands, and arms surfaces. Healing occurs with atrophic scars. Toe nails are dystrophic, but finger nails are normal. The patient has normal hair and no dental abnormalities and shows no mucosal involvement. Immunofluorescence mapping of perilesional skin biopsy specimen with antibodies against the cutaneous basement membrane proteins showed an epidermal separation within the lamina lucida of the skin and reduced staining for the laminin-332 chains favoring the diagnosis of Junctional epidermolysis bullosa to laminin 332. In March 2019 the patient was admitted to the General and Transplantation Surgery Department (at the Medical University of Warsaw Central Teaching Hospital) with a long-standing persistent ulceration on her right leg induced by mechanical trauma. During her stay, the patient underwent physical and psychological examination, including a complete review of her medical history and medical records, followed by consultation of an interdisciplinary team of dermatologists, surgeons, biotechnologists, and pathologists, and the patient was qualified to receive treatment with an innovative surgical procedure with the use of a new biological dressing.

During initial exam a wound swab culture produced *Enterobacter cloacae*, *Acinetobacter pittii*, and *Staphylococcus aureus*. Initial medical treatment involved sensitivity-test-based

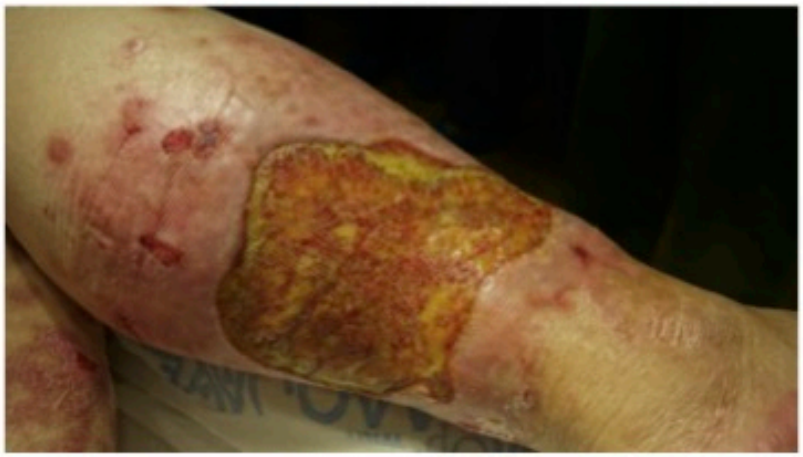

Fig 1. Day 0, admission, 15×15 cm, fibrin-covered ulceration, located on the posterolateral surface of the patient's right leg accompanied with multiple postulcerative trophic lesions on the distal segment of the left leg, isolated bullae, multiple dispersed erosions covered with erythematous skin.

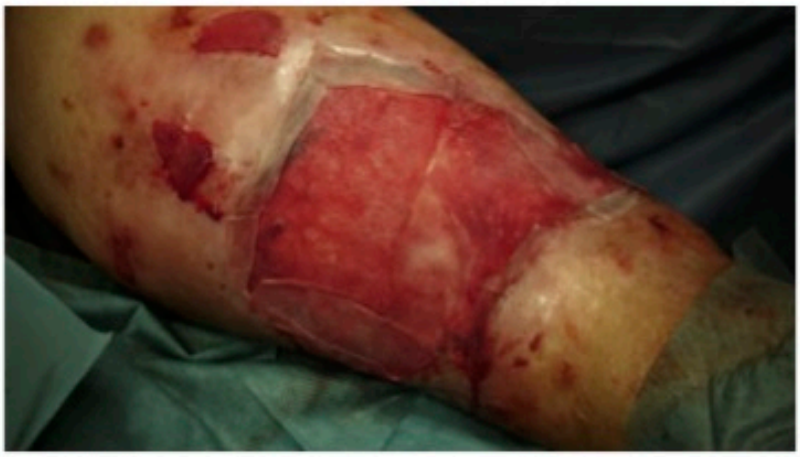

Fig 2. Day 0, procedure, wound covered with prepared graft.

antibiotic therapy with moxifloxacin (at 400 mg once daily) and clindamycin (at 1 g/day) and anticoagulation prophylaxis with enoxaparin at 100 mg/day. A clinical examination conducted on admission revealed a 15×15 cm, fibrin-covered ulceration, located on the posterolateral surface of the patient's right leg (Fig 1). There were also multiple postulcerative trophic lesions on the distal segment of the left leg, isolated bullae, multiple dispersed erosions covered with erythematous skin, plantar hyperkeratosis, dystrophic finger- and toenails, and enamel hypoplasia due to the underlying disease.

The patient underwent a surgical procedure, during which the ulceration was debrided with a water-knife. Subsequently, a radiation-sterilized acellular allogenic skin graft was soaked in a sterile solution of physiological saline for 5 to 10 seconds. The skin graft was cut to size with sterile scissors and placed over the ulcerated area of the skin in such a way as to achieve maximum tissue contact (Fig 2). The allogenic graft (biological dressing) was then covered with a nonadhesive Bactigras dressing and 3 layers of bandage to ensure a tight fit.

The first dressing change was conducted on postoperative day 5. By then the graft was viable and showed a pinkish tinge. The ulceration covered with the biological dressing was gently rinsed, first with Octenisept (octenidine dichydrochloride + phenoxyethanol), then with sterile physiological saline, and finally covered with a Bactigras dressing. On postoperative day 7, skin biopsy samples were collected for histopathological examination with immunofluorescent staining. Wound healing was assessed based on photographic documentation, which included photographs taken prior to surgery and 1 day, 30 days, and 6 months post-surgery (Fig 3).

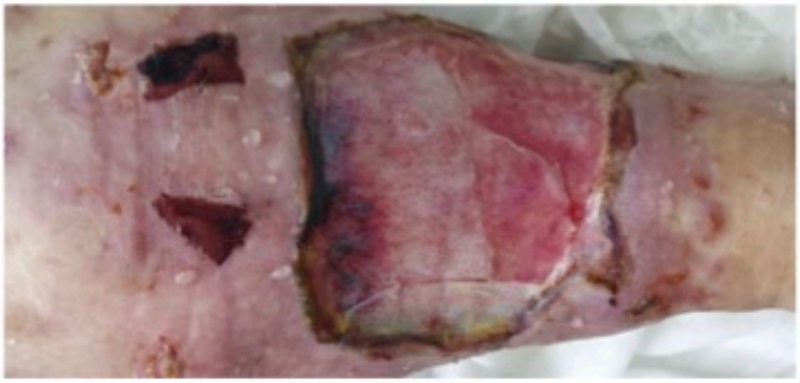

Fig 3. Day 30, dressing change.

RESULTS

Our data indicates the safety and efficacy of grafting this novel medicinal product in patients with rare disorders, such as EB. The 6-month follow-up demonstrated the safety of using a human-derived dermal scaffolding with no visible evidence of infection or necrosis. We observed beneficial effects on wound healing, a reduced number of required dressing changes, reduced pain, and an overall improvement in the EB patient's quality of life.

DISCUSSION

The term epidermolysis bullosa refers to a heterogeneous group of conditions characterized by skin fragility and markedly diverse phenotypes and clinical outcomes. One common diagnostic feature of this group of conditions is the formation of bullae on the skin following relatively insignificant injuries. These bullae lead to erosions and nonhealing ulcerations [1]. In some subtypes of EB these lesions lead to disfiguring scars and early development of invasive squamous cell carcinoma [13,14]. Despite the immense progress in the understanding of molecular genetics and the underlying pathologic mechanisms of EB that had taken place over the last several decades, there is still no cure for EB.

There have been a number of preclinical attempts at developing novel treatment methods for EB. The goal of these treatment attempts was to correct the primary genetic defect at the DNA, mRNA, or protein level with the use of 1. induced pluripotent stem cells or keratinocyte-based gene therapy [15,16], 2. antisense oligonucleotide therapy [17], and 3. drugs inducing premature termination codon read-through [18,19]. Subsequent, potential lines of treatment involve medications modifying the course of the disease by reducing symptoms and targeting the inflammatory and fibrotic processes responsible for specific EB phenotypes. Despite the fact that such reports are very promising, all potentially effective EB therapies are currently evaluated as part of preclinical studies, and it will be a long time before they are marketed [10,20–22]. Thus, searching for novel alternatives in the treatment of EB continues to be very important.

CONCLUSION

Our clinical study data demonstrated the safety and efficacy of the evaluated allograft in a patient with a rare disease, namely EB.

As of now, the protocol for allogenic acellular epidermal sheet grafting is the most promising treatment method of severely affected skin areas in EB patients. Moreover, this treatment shows potential in preventing the formation of bullae and the associated complications, such as cancer. The first 200 days of follow-up demonstrated the safety of using human skin scaffolds. We observed beneficial effects of treatment and an improved quality of life in the EB patient. Despite the fact that the evaluated surgical protocol has only been used in 1 patient so far, we hope that this case report will motivate others to take part in the discussion and further studies on this topic, which in turn may contribute to radiation-sterilized allogenic acellular skin grafts being approved for use as potential biological dressings to develop new medicinal products in EB patients.

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Rozdział IV Podsumowanie wyników badań i wnioski końcowe

1. Wyniki przedstawione w badaniach *in vitro* i *in vivo* potwierdzają bezpieczeństwo produktu ATMP.
2. Badania immunohistochemiczne, histologiczne, mikroskopia elektronowa i konfokalna potwierdziły i wykazały naciek komórek gospodarza i neowaskularyzację opatrunku biologicznego. Ponadto opatrunki charakteryzowały się niską immunogennością, potwierdzoną badaniami histologicznymi i proliferacją limfocytów T *in vitro*.
3. Wyniki badań bezpośrednie i odległe (powyżej 3 miesięcy) potwierdzają jednoznacznie skuteczność terapeutyczną opatrunku biologicznego w postaci bezkomórkowej macierzy zasiedlonej komórkami macierzystymi.
4. Przeszczepienie opatrunku biologicznego BIOOPA zdecydowanie przyczyniło się do zmniejszenia powierzchni rany lub całkowitego wygojenia się rany. Zmniejszenie/zniesienie/redukcja bólu i świądu miała istotny wpływ na poprawę jakości życia u pacjentów.

Załączniki

Oświadczenie Komisji Bioetycznej przy Warszawskim Uniwersytecie Medycznym

Komisja Bioetyczna
przy Warszawskim Uniwersytecie Medycznym

Tel.: 022/ 57 - 20 -303
Fax: 022/ 57 - 20 -165

ul. Żwirki i Wigury nr 61
02-091 Warszawa

e-mail: komisja.bioetyczna@wum.edu.pl
www.komisja-bioetyczna.wum.edu.pl

KB/.....177...../2015

Komisja Bioetyczna przy Warszawskim Uniwersytecie Medycznym
po zapoznaniu się z wnioskiem /wymienić wnioskodawcę/ - **w dniu 08 września 2015r.**
Prof. dr hab. med. Cezary Kowalewski, Klinika Dermatologii i Immunodermatologii WUM,
ul. Koszykowa 82a, 02-008 Warszawa

dotyczącym: wyrażenia opinii w sprawie badania pt. „Opracowanie innowacyjnej metody leczenia Epidermolysis Bullosa oraz ran przewlekłych innego pochodzenia za pomocą opatrunku biologicznego z materiału ludzkiego"

wyraża następującą
o p i n i ę

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

Uwagi Komisji-verte

Pouczenie-w ciągu 14 dni od otrzymania decyzji wnioskodawcy przysługuje Prawo odwołania do Komisji Odwoławczej za pośrednictwem Komisji Bioetycznej przy Warszawskim Uniwersytecie Medycznym.
Komisja działa na podstawie art.29 ustawy z dnia 5.12.1996r. o zawodzie lekarza /Dz.U.nr 28/97 poz.152 wraz z późn.zm./, zarządzenia MZiOS z dn.11.05.1999r. w sprawie szczegółowych zasad powoływania i finansowania oraz trybu działania komisji bioetycznych /Dz.U.nr 47 poz.480/, Ustawy prawo farmaceutyczne z dnia 6 września 2001r. (Dz.U.Nr 126, poz. 1381 z późn. zm.) Zarządzenie nr 56/2007 z dnia 15 października 2007 r.w sprawie działania Komisji Bioetycznej przy Warszawskim Uniwersytecie Medycznym /Regulamin Komisji Bioetycznej przy Warszawskim Uniwersytecie Medycznym/.
Komisja działa zgodnie z zasadami GCP.
W załączeniu - skład Komisji oraz lista obecności.

Przewodnicząca
Komisji Bioetycznej

Prof. dr hab. n. med. Maria Roszkowska-Blaim

*niepotrzebne skreślić

Komisja Bioetyczna
przy Warszawskim Uniwersytecie Medycznym

Tel.: 022/ 57 - 20 -303
Fax: 022/ 57 - 20 -165

ul. Żwirki i Wigury nr 61
02-091 Warszawa

e-mail: komisja.bioetyczna@wum.edu.pl
www.komisja-bioetyczna.wum.edu.pl

KB/19/A2020

Komisja Bioetyczna przy Warszawskim Uniwersytecie Medycznym
w dniu 09 marca 2020 r. po zapoznaniu się z wnioskiem:

Prof. dr hab. n. med. Cezary Kowalewski
Klinika Dermatologii i Wenerologii,
ul. Koszykowa 82 a , 02-008 Warszawa,

dotyczącym: akceptacji zmian w dokumentacji obejmujących:

- Kopia dokumentu potwierdzającego zawarcie umowy obowiązkowego ubezpieczenia odpowiedzialności cywilnej Sponsora i Badacza, obejmującej cały okres trwania badania klinicznego, za szkody wyrządzone w związku z prowadzeniem badania klinicznego – Polisa Ubezpieczeniowa z dnia 12.10.2018
- Kopia protokołu badania, wersja 2.0 z dnia 31.10.2019
- Streszczenie Protokołu badania w języku polskim, wersja 2.0 z dnia 31.10.2019
- Kopia Broszury Badacza, wersja 2.0 z dnia 31.10.2019
- Wykaz Ośrodków Badawczych z dnia 31.10.2019
- Wzór Karty Obserwacji Klinicznej dla pacjentów chorych na EB w języku angielskim, wersja 2.0 z dnia 30.10.2019
- Wzór Karty Obserwacji Klinicznej dla pacjentów z owrzodzeniami żylnymi w języku angielskim, wersja 2.0 z dnia 30.10.2019
- Wzór Karty Obserwacji Klinicznej dla pacjentów z ranami oparzeniowymi w języku angielskim, wersja 2.0 z dnia 30.10.2019
- Wzór Informacji dla Pacjenta i Formularz Świadomej Zgody na Udział w Badaniu Klinicznym, uczestnika badania klinicznego dla chorych na EB dla:
  - Pacjentów 5-11 lat, wersja 2.0 z dnia 31.10.2019
  - Pacjentów 12-15 lat, wersja 2.0 z dnia 31.10.2019
  - Pacjentów 16-17 lat, wersja 2.0 z dnia 31.10.2019
  - Pacjentów >18 lat, wersja 2.0 z dnia 31.10.2019
  - Rodziców lub przedstawiciela ustawowego Pacjenta, wersja 2.0 z dnia 31.10.2019
- Wzór Informacji dla Pacjentki/Pacjenta i Formularz Świadomej Zgody na Udział w Badaniu Klinicznym, Formularz Zgody na przetwarzanie danych osobowych uczestnika badania klinicznego, dla pacjentów z ranami oparzeniowymi, wersja 2.0 z dnia 31.10.2019
- Wzór Informacji dla Pacjentki/Pacjenta i Formularz Świadomej Zgody na Udział w Badaniu Klinicznym, Formularz Zgody na przetwarzanie danych osobowych uczestnika badania klinicznego, dla pacjentów z owrzodzeniami żylnymi, wersja 2.0 z dnia 31.10.2019

Oświadczenia współautorów publikacji

Prof. dr hab. n. med. Cezary Kowalewski
Klinika Dermatologii i Wenerologii
UCK WUM

OŚWIADCZENIE

W związku z ubieganiem się lek. Magdaleny Nita o stopień doktora nauk medycznych oświadczam, że jestem współautorem publikacji pt. ***„ New Treatment of Wound healing with allogenic acellular human skin graft: preclinical assessment and in vitro study".*** Oświadczam , że mój procentowy wkład autorski wynosi 1%. Mój udział w przygotowaniu tej publikacji polegał na merytorycznym wsparciu.

Warszawa, 8.10.2021
(miejscowość, rok – miesiąc - dzień)

Prof. dr hab. n. med.
CEZARY KOWALEWSKI
specjalista dermatolog
(czytelny podpis)

Prof. dr hab. n. med. Cezary Kowalewski
Klinika Dermatologii i Immunodermatologii
UCK WUM

OŚWIADCZENIE

W związku z ubieganiem się lek. Magdaleny Nita o stopień doktora nauk medycznych oświadczam, że jestem współautorem publikacji pt. ***„Transplantation of a New Biological Product in Rare Diseases, Such as Epidermolysis Bullosa: Response and Clinical Outcome".*** Oświadczam , że mój procentowy wkład autorski wynosi 1%. Mój udział w przygotowaniu tej publikacji polegał na merytorycznym wsparciu.

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(miejscowość, rok – miesiąc - dzień)

.....
(czytelny podpis)

Prof. dr hab. n. med. Cezary Kowalewski
Klinika Dermatologii i Wenerologii
UCK WUM

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Prof. dr hab. n. med. CEZARY KOWALEWSKI Specjalista dermatolog

Warszawa, 8.12.2021
(miejscowość, rok – miesiąc - dzień)

Cezary Kowalewski
(czytelny podpis)

Dr hab.Andrzej Eljaszewicz
Zakład Medycyny Regeneracyjnej I Immunoregulacji
Uniwersytet Medyczny w Białymstoku

OŚWIADCZENIE

W związku z ubieganiem się lek. Magdaleny Nita o stopień doktora nauk medycznych oświadczam, że jestem współautorem publikacji pt. ***„Epidermolysis Bullosa (EB)–safe and effective therapy in the light of clinical trials. New Approach to treatment by innovative method (BIOOPA-ATMP medicinal products)".*** Oświadczam , że mój procentowy wkład autorski wynosi 1%. Mój udział w przygotowaniu tej publikacji polegał na merytorycznym wsparciu.

Białystok 2021/10/01
(miejscowość, rok – miesiąc - dzień)

(czytelny podpis)

hab.Andrzej Eljaszewicz
Zakład Medycyny Regeneracyjnej I Immunoregulacji
Uniwersytet Medyczny w Białymstoku

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Białystok 2021/10/01
(miejscowość, rok – miesiąc - dzień)

(czytelny podpis)

Dr hab.Andrzej Eljaszewicz
Zakład Medycyny Regeneracyjnej I Immunoregulacji
Uniwersytet Medyczny w Białymstoku

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Białystok 2021/10/01
(miejscowość, rok – miesiąc - dzień)

(czytelny podpis)

prof. dr hab. n. med. Piotr Fiedor
Klinika Chirurgii Ogólnej i Transplantacyjnej
UCK WUM SKDJ

OŚWIADCZENIE

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Warszawa, 07.10.2021r.
(miejscowość, rok – miesiąc - dzień)

.....
(czytelny podpis)

prof. dr hab. n. med. Piotr Fiedor
Klinika Chirurgii Ogólnej i Transplantacyjnej
UCK WUM SKDJ

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(czytelny podpis)

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Klinika Chirurgii Ogólnej i Transplantacyjnej
UCK WUM SKDJ

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prof. dr hab. n. med. Piotr Fiedor
Klinika Chirurgii Ogólnej i Transplantacyjnej
UCK WUM SKDJ

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(miejscowość, rok – miesiąc - dzień)

.....
(czytelny podpis)

Dr n. med. Jacek Pliszczyński
Klinika Chirurgii Ogólnej i Transplantacyjnej
UCK WUM Szpital Kliniczny Dzieciątka Jezus

OŚWIADCZENIE

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.....
(czytelny podpis)

Dr n. med. Jacek Pliszczyński
Klinika Chirurgii Ogólnej i Transplantacyjnej
UCK WUM SKDJ

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.....
(miejscowość, rok – miesiąc - dzień)

Dr n. med. Jacek Pliszczyński
Specjalista Chirurgii Ogólnej
27.12.06

.....
(czytelny podpis)

Dr n. med. Jacek Pliszczyński
Klinika Chirurgii Ogólnej i Transplantacyjnej
UCK WUM SKDJ

OŚWIADCZENIE

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.....
(miejscowość, rok – miesiąc - dzień)

Dr n. med. Jacek Pliszczyński
Specjalista
Chirurgii Ogólnej
15.06.

.....
(czytelny podpis)

Dr n. med. Jacek Pliszczyński
Klinika Chirurgii Ogólnej i Transplantacyjnej
UCK WUM SKDJ

OŚWIADCZENIE

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.....
(miejscowość, rok – miesiąc - dzień)

dr hab. n. med. Artur Marek Kamiński
Zakład Transplantologii i Centralny Bank Tkanek
UCK WUM

OŚWIADCZENIE

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Warszawa 2021-10-06
(miejscowość, rok – miesiąc - dzień)

(czytelny podpis)

Dr hab. Artur Marek Kamiński
Zakład Transplantologii i Centralny Bank Tkanek

OŚWIADCZENIE

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Warszawa, 2021-10-06
(miejscowość, rok – miesiąc - dzień)

(czytelny podpis)

Dr hab. Artur Marek Kamiński
Zakład Transplantologii i Centralny Bank Tkanek
WUM

OŚWIADCZENIE

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Warszawo 2020.10.06
(miejscowość, rok – miesiąc - dzień)

(czytelny podpis)

Prof. dr hab. Katarzyna Woźniak
Klinika Dermatologii i Immunodermatologii
UCK WUM

OŚWIADCZENIE

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Warszawa 8 X 2021 ..... Katarzyna Woźniak

(miejscowość, rok – miesiąc - dzień) (czytelny podpis)

Prof. dr hab. n. med. Katarzyna Woźniak
Klinika Dermatologii i Immunodermatologii
UCK WUM

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Warszawa 8x 2021
.....
(miejscowość, rok – miesiąc - dzień)

Katarzyna Woźniak
.....
(czytelny podpis)

Prof. dr hab. n. med. Katarzyna Woźniak
Klinika Dermatologii i Immunodermatologii
UCK WUM

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Wwe, 8X 2014
(miejscowość, rok – miesiąc - dzień)

Katarzyna Woźniak
(czytelny podpis)

Prof. dr hab. Marcin Moniuszko
Zakład Medycyny Regeneracyjnej I Immunoregulacji
Uniwersytet Medyczny w Białymstoku

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Podpisano przez:

Marcin Moniuszko

Date / Data:
2021-10-08 16:16

.....
(miejscowość, rok – miesiąc - dzień)

.....
(czytelny podpis)

Prof. dr hab. Marcin Moniuszko
Zakład Medycyny Regeneracyjnej I Immunoregulacji
Uniwersytet Medyczny w Białymstoku

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Signed by /
Podpisano przez:

Marcin Moniuszko

Date / Data:
2021-10-08 16:17

.....
(miejscowość, rok – miesiąc - dzień)

.....
(czytelny podpis)

Prof. dr hab. Marcin Moniuszko
Zakład Medycyny Regeneracyjnej I Immunoregulacji
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.....
(czytelny podpis)

Dr n. med. Tomasz Ołdak
Polski Bank Komórek Macierzystych

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Warszawa, 2021/10/08
(miejscowość, rok - miesiąc - dzień)

Tomasz Ołdak
(czytelny podpis)

prof. dr hab. n. med. Maciej Kosieradzki
Klinika Chirurgii Ogólnej i Transplantacyjnej
UCK WUM SKDJ

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Dr hab. n. med. Maciej Kosieradzki

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KIEROWNIK KLINIKI
Chirurgii Ogólnej i Transplantacyjnej
Dr hab. n. med. Maciej Kosieradzki

.....
(czytelny podpis)

prof. dr hab. n. med. Maciej Kosieradzki
Klinika Chirurgii Ogólnej i Transplantacyjnej
UCK WUM SKDJ

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Dr hab. n. med. Maciej Kosieradzki

.....
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prof. dr hab. n. med. Maciej Kosieradzki
Klinika Chirurgii Ogólnej i Transplantacyjnej
UCK WUM SKDJ

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ORDYNATOR
Chirurgii Ogólnej i Transplantacyjnej
Dr hab. n. med. Maciej Kosieradzki
(czytelny podpis)

prof. dr hab. n. med. Sławomir Majewski
Klinika Dermatologii i Wenerologii
UCK WUM

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prof. dr hab. n. med. Sławomir Majewski
Klinika Dermatologii i Wenerologii
UCK WUM

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(czytelny podpis)

dr Zbigniew Zimek
Instytut Chemii i Technologii Jądrowej
Politechnika Warszawska

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WARSZAWA 2021 10 08
(miejscowość, rok - miesiąc - dzień)

.....
(czytelny podpis)

dr Zbigniew Zimek
Instytut Chemii i Technologii Jądrowej
Politechnika Warszawska

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Warszawa 2021.10.08
(miejscowość, rok - miesiąc - dzień)

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