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The healing dynamics of non-healing wounds using cryo-preserved amniotic membrane

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Abstract

We evaluated the effect of the application of cryo-preserved amniotic membrane on the healing of 26 non-healing wounds (18 patients) with varying aetiologies and baseline sizes (average of 15.4 cm^2), which had resisted the standard of care treatment for 6 to 456 weeks (average 88.8 weeks). Based on their average general responses to the application of cryo-preserved AM, we could differentiate three wound groups. The first healed group was characterised by complete healing (100% wound closure, maximum treatment period 38 weeks) and represented 62% of treated wounds. The wound area reduction of at least 50% was reached for all wounds in this group within the first 10 weeks of treatment. Exactly 19% of the studied wounds responded partially to the treatment (partially healed group), reaching less than 25% of closure in the first 10 weeks and 90% at maximum for extended treatment period (up to 78 weeks). The remaining 19% of treated wounds did not show any reaction to the AM application (unhealed defects). The three groups have different profiles of wound area reduction, which can be used as a guideline in predicting the healing prognosis of non-healing wounds treated with a cryo-preserved amniotic membrane.

Key Messages

- we evaluated the effect of the application of cryo-preserved amniotic membrane on the healing of non-healing wounds
- twenty-six wounds (18 patients) of various aetiologies and baseline sizes with a history of long preceding resistance to standard care were treated for an extended time period (up to 78 weeks)
- we showed that the amniotic membrane application had a profitable effect promoting a complete healing of 62% of treated, previously non-healing defects

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- analysis of our results also suggests that the dynamics of healing process can be used as a predictor of the outcome of the treatment

KEYWORDS

cryo-preserved amniotic membrane, healing dynamics, non-healing wounds

1 | INTRODUCTION

The general term 'chronic wounds' envelopes a heterogeneous group of wounds exhibiting specific healing process physiology different from the acute ones. They typically require a considerably long healing time and are characterised by tissue renewal by granulation.[1] The period required for a wound to be classified as chronic has been defined in the range of 4 weeks up to more than 3 months.[2] As the nomenclature 'chronic wound' is not clear, the European Wound Management Association (EWMA) proposed to use the term 'non-healing wounds' (NHWs),[3] which will be used herein. Common features of NHW include persistent infections, the formation of drug-resistant microbial biofilms, and loss of dermal/epidermal cells ability to respond to reparative stimuli.[4] NHWs are associated with numerous pathological conditions: diabetes mellitus (DM), peripheral artery disease (PAD), chronic venous insufficiency, post-traumatic wounds, or postsurgical wound dehiscence. Apart from the pathological effects, the NHWs have an important impact on the quality of life of affected subjects in the sense of elimination or discrimination from society, limited mobility, and productivity.[5]

The current standard of care (SOC) for treating NHW includes surgical debridement, infection control, appropriate dressing, and treatment of primary pathology. Despite the development of many types of wound dressings, the healing of NHW is often challenging to achieve. Using SOC, closure rates for NHW range from 21% to 35%, and the recurrence rate is high.[6] The increase in the healing efficiency and reduction of the recurrence rate, the healing time, and the costs are thus the objectives of the new approaches to NHW care. The use of different forms of biological dressings, particularly placental derivatives, has been accepted as a promising tool in regenerative medicine.[7-10] Most frequently, they are based on amniochorionic (ACM) or amniotic membrane (AM). For decades the ACM and AM have been recognised for their wound healing stimulation properties and their negligible immunogenicity, making them an attractive choice for biological wound dressing. AM application has been adapted particularly in the ocular surface healing, and in the last decade, its efficiency is broadly confirmed in other surgical procedures in dermatology, plastic surgery, genitourinary medicine, and otolaryngology.[8,11-13]

AM application promotes several effects supporting wound healing, namely anti-inflammatory, anti-fibrotic, anti-microbial, neurotrophic, and analgesic. Besides that, AM influences angiogenesis. AM grafting does not lead to immunological rejection, and there is no need for any immunosuppressive treatment.[13-16] Therefore, the AM grafts are considered a safe substrate, promoting proper granulation and epithelisation, assuring better hydration of the wound bed while suppressing excessive fibrosis. Many studies have reported the healing benefits of AM applied to NHW; improved healing rates (percentage of completely healed wounds) and significantly shortened healing times have been documented.

Systematic studies of the effect of the AM or ACM effect date back to the early 80s of the last century.[17] Often such studies report different healing success rates and wound closure progress, depending on the procedure of AM or ACM preparation (cryo-preserved, dried, or lyophilised),[18] AM application frequency,[19] type of wound treated (diabetic, venous, or arterial ulcer, surgical wound, dehiscence),[20] and the treatment and application approach selected.[7,21,22]

The complete healing rate of NHW because of AM's beneficial effect is reported to be anywhere between 20%[23] and 100%,[24] indicating the existence of non-negligible proportions of subjects who will not respond to the AM treatment. However, it is still unclear whether it is possible to identify such individuals already at the early treatment period (although some indications exist[25]). Because of the elevated cost of AM treatment, this information can be an important economic factor when opting for AM application after SOC failure.

In the present work, we studied the effect of cryo-preserved AM application on NHW of various aetiologies (venous, arterial, postoperative, diabetic) and various sizes (from 0.5 to 98 cm^2), which had previously resisted SOC for more than 6 weeks (average 88.8). By evaluating the healing progress, we aimed to determine the efficiency of the AM treatment and analyse whether the dynamics of the wound closure can be used as a predictor/estimator for the efficacy of the AM treatment of NHW.

2 | MATERIALS AND METHODS

The study followed the Ethics Committee standards of four participating institutions (1st Medical Faculty of Charles University, General University Hospital, University Hospital Motol, and Na Homolce Hospital, all in Prague) and adhered to the tenets set out in the Declaration of Helsinki.

3 | SUBJECTS

The patients for the study were selected according to the following criteria: Inclusion criteria: age ≥18 years, the presence of resistant NHW with the duration of more than 6 weeks, wound of maximum size of 150 cm^2 extending through the full thickness of the skin but not reaching to the tendon or bone. Exclusion criteria: Tendon or bone exposure in the wound, allergy to antibiotics used in solution for AM decontamination, transcutaneous oximetry value below 30 mmHg for patients with DM, known history of AIDS or HIV, ankle brachial index (ABI) <0.5, for all patients except those with DM, suspicious for cancer or history of radiation at wound site, severe (uncontrolled) systemic disease, or planned surgical intervention in the next 6 months.

Patients willing to participate in the study and complying with the criteria signed an informed consent form. Eighteen patients were enrolled in the study (13 men, 5 women) with a total of 26 wounds (multiple wounds in some patients). The average age of the patients was 62.6 years (26 to 85). The treated wound size varied between 0.5 and 98 cm^2 with an average of 15.4 ± 20.2 cm^2. The wound resistance to treatments preceding the enrolment into the study spanned from 6 to 456 weeks with an average of 88.8 weeks. The demographic data of patients are summarised in Table 1.

4 | AM GRAFTS PREPARATION

All placenta donors signed informed consent and were checked negative for hepatitis B and C, syphilis, and HIV, and C-reactive protein was < 20 mg/L. Placentas were obtained from full-term deliveries by elective caesarean section in the Motol University Hospital, Prague, and before processing visually inspected for injuries, visible pathologies, or comorbidities. Then they were immediately placed in a sterile container, overlaid with decontamination solution BASE•128 (Alchimia, Ponte San Nicolò, Italy), and stored at room temperature for 20 hours. The placentas were subsequently processed in aseptic conditions. They were repeatedly cleansed using physiological solution (0.9% NaCl), AM sheets were peeled off by blunt dissection, and blood clots were removed. AM was then overlaid with DMEM (c.n. 32 430 027, Thermo Fisher Scientific) supplemented with antibiotics (Piperacillin/Tazobactam, Amphotericin B, Vancomycin, and Gentamicin) and stored for at least 2 hours to complete the decontamination. Next, the AM sheets were rinsed in physiological solution, stretched on Sanatyl support (Tylex, Letovice, Czech Republic), and sectioned into patches of the desired size. Finally, AM pieces were placed in containers filled with storage medium (50% glycerol in DMEM) (glycerol, Dr Kulich Pharma, Czech Republic) and stored at −80°C. Tissues with negative microbiology tests and negative repeated serology examination performed after 6 months were released after 6 months for grafting.

5 | AM DRESSING APPLICATION AND THE WOUND TREATMENT PROCEDURE

All patients followed up a standard visit protocol. The study visits were scheduled every 7 days and included photodocumentation of the healing process, evaluation of subjective pain perception and completion of patients quality of life questionnaire, the treatment of the wound and surrounding skin, application of AM, and secondary fixation dressing application. The initial AM application frequency was set to weekly (every visit), but it was modulated later according to the evolution of the healing progress. Wound care procedure was standardised for all centres similarly: wound debridement, rinsing with saline, cultivation collection (each 4 weeks, or if necessary), and disinfectant solution application. After thawing, the AM was removed from the storage solution and washed with saline (2 × 5 minutes) in a sterile container. The AM graft was applied with at least 5 mm overlap, and complete contact with the wound surface was assured. After verifying appropriate graft adhesion, a fixation with foam cover (Mepilex XT, Mölnlycke Health, Sweden) was formed with an overlap of at least 2 cm and fixed with a bandage. In patients with venous insufficiency, a compression bandage was added. Wound dressing was left for 3 to 5 days, depending on the condition of the wound. In case of need of redressing at home, the patients were equipped with the necessary material and were eventually assisted by home care agency.

6 | HEALING PROCESS EVALUATION

The wound healing progress was regularly monitored during the scheduled visits for wound treatment and

TABLE 1 Demographic data

Patient number	Age	Sex	DM	Smoker	Comorbidities	Defect number	Location	Aetiology
P1	77	M	N	N	atrial fibrillation	D1	right calf	venous
P2	60	M	Y	N	hypertension, hyperlipidaemia, renal insufficiency, atrial fibrillation, st.p. AVR	D2	left calf	venous
						D3	left calf	venous
P3	72	M	N	N	hypertension, atrial fibrillation, renal insufficiency	D4	left calf	venous
						D5	left calf	venous
						D6	left calf	venous
						D7	left calf	venous
P4	64	M	N	N	renal insufficiency	D8	right calf	venous
						D9	right calf	venous
P5	72	M	N	N	hypertension	D10	right ankle	venous/ arterial
						D11	right ankle	venous/ arterial
P6	56	F	N	Y	hypertension, peripheral artery disease	D12	left calf	defect after fasciotomy
P7	33	M	N	Y	hypertension	D13	right lower leg	venous
P8	60	M	N	N	hypertension	D14	right leg	venous
P9	66	F	N	Y	hypertension	D15	left ankle	venous
P10	65	M	Y	N	hypertension	D16	right leg	arterial
P11	85	F	Y	N	hypertension, peripheral arterial disease, anaemia	D17	left ankle	venous
P12	26	F	N	N	hypertension, hyperlipidaemia, st.p. AVR, st.p. CABG	D18	sternum	dehiscence
P13	45	M	N	N	hypertension	D19	left ankle	venous
						D20	left ankle	venous
P14	68	F	Y	N	X	D21	right ankle	venous
P15	67	M	Y	N	hypertension, hyperlipidaemia	D22	left calf	venous
						D23	left calf	venous
P16	67	M	N	N	X	D24	right ankle	venous
P17	74	M	Y	Y	hypertension, peripheral artery disease, chronic renal failure, anaemia, st.p. CABG	D25	left calf	defect after fasciotomy
P18	69	M	N	Y	hypertension	D26	left ankle	arterial

Abbreviations: DM, diabetes mellitus; st.p. AVR, status post aortic valve replacement; st.p. CABG, status post coronary artery bypass grafting.

dressing renewal. The wound size and the state were photo-documented with a scale indicating the patient ID number, the defect number, and the visit date placed in the closest proximity of the wound to assure the image unique identifier and proper image scaling. The wound size was determined by manual tracing of the wound border on calibrated images with automatic determination of the area size using NIS-Elements software (Laboratory Imaging, The Czech Republic). Wound area reduction, WAR = 100*(Baseline size − Current size)/Baseline size): the parameter was used to evaluate the healing progress, expressing the wound area's percentage at a given treatment period relative to the original size.

The wound was considered as healed (H) only when 100% reepithelialisation was achieved. Wounds with WAR between 50 and 99% were considered as partially healed (PH). Not achieving a reduction in the area by at least 50% was judged as failure to heal (unhealed defects [UH]), in accordance with the most often used criterion.[26] The average wound closure profiles for the H and PH group were fitted with asymptotic function ($y = a - b.c^x$) using ORIGIN50 software. For the unhealed (UH) group, only an approximate linear fit was performed because of the scattered character of the average.

Other than the wound itself, the perception of pain related to the wound was also monitored. The pain

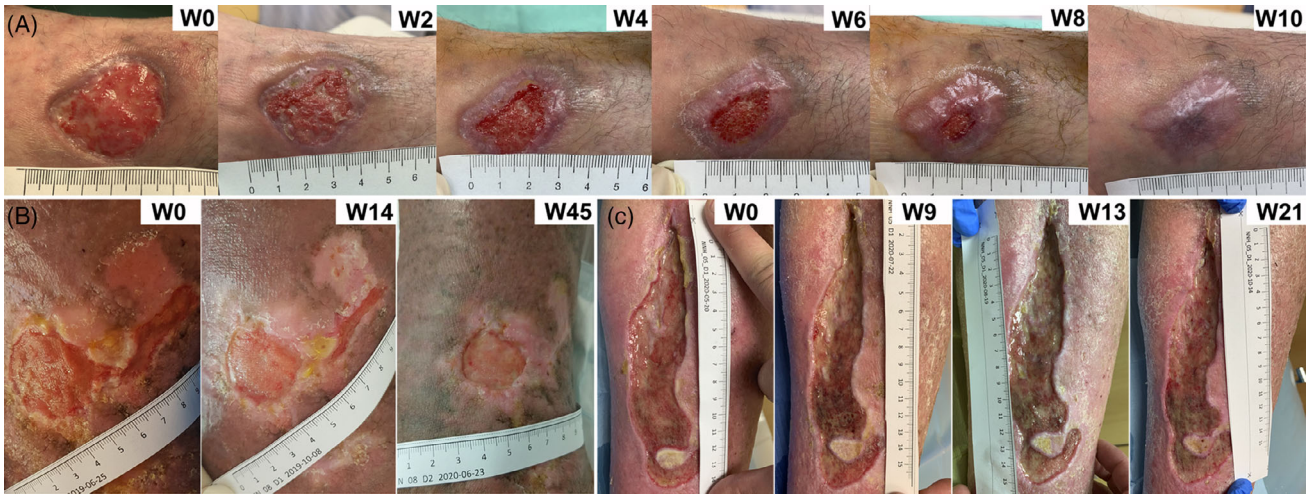

FIGURE 1 Examples of wound closing. A, healed (H) wound (D15, venous leg ulcer); B, partially healed (PH) wound (D19, venous leg ulcer); C, wound with no reaction (D25, defect after fasciotomy). W0: the wound state after 24, 456, and 100 weeks of outpatient care with SOC treatment for A, B, and C, respectively. W: number of weeks of treatment with AM

TABLE 2 Defects' characteristics and outcome data

Patient number	Defect number	Time from onset (w)	Baseline size (cm^2)	Treatment duration (w)	Wound closure (%)	End status	Number of visits	Number of AM applications
P1	D1	315	2.6	9	100	H	10	9
P2	D2	36	16.8	32	100	H	31	15
	D3	36	4.5	17	100	H	17	11
P3	D4	7	28.1	16	100	H	15	11
	D5	7	1.0	6	100	H	5	2
	D6	7	2.4	6	100	H	6	4
	D7	7	13.8	36	100	H	36	15
P4	D8	13	6.2	31	100	H	31	14
	D9	13	2.9	35	100	H	31	15
P5	D10	6	1.3	12	100	H	11	4
	D11	250	5.2	38	100	H	37	19
P6	D12	6	48.7	33	100	H	29	29
P7	D13	53	5.3	36	100	H	34	30
P8	D14	12	0.5	14	100	H	14	14
P9	D15	24	10.7	13	100	H	8	8
P10	D16	8	6.0	5	100	H	6	4
P11	D17	52	98.0	74	79	PH	74	69
P12	D18	20	14.4	44	50	PH	38	32
P13	D19	456	22.9	78	64	PH	67	46
	D20	456	13.9	78	74	PH	67	45
P14	D21	50	7.1	49	90	PH	39	39
P15	D22	117	30.5	6	−28	UH	6	4
	D23	117	5.6	11	18	UH	11	9
P16	D24	100	5.0	19	−3	UH	19	12
P17	D25	100	33.6	18	−22	UH	13	9
P18	D26	40	12.7	17	−34	UH	13	11

level was evaluated using the 0 to 10 scale, 0 equal none, 10—unbearable.

7 | RESULTS

Of 18 patients (26 defects), 10 responded to the AM treatment by complete healing of all wounds (H group, 16 defects, 62%). Four patients (five defects, 19%) exhibited partially positive limited response (PH group). They reached on average 71.4 ± 13.6% of WAR and never exceeded 90% wound closure despite, in some cases, significantly prolonged treatment (up to 74 weeks). They showed less than 20% improvement over the period of first 9 to 20 weeks. Four patients (five defects, 19%) did not react to the treatment at all and were assigned to the unhealed group (UH group). In no case, an adverse secondary reaction to the AM application was observed. In cases when the defect responded well to the AM treatment, the epithelisation onset was very fast (after 2 AM applications), and the wound closure progressed rapidly towards complete healing. Typical wound healing in the course of treatment (D16) is presented in Figure 1A.

The period required for the defect's complete healing in the H group varied from 5 to 38 weeks (average 21.2 ± 12.2, median 16.5) and 2 to 30 AM applications (12.8 ± 7.9, 12.5) were needed. The WAR progress in the PH group was significantly slower, and its average showed the tendency to converge to a maximum value of approximately 88%. The data summarising outcomes for

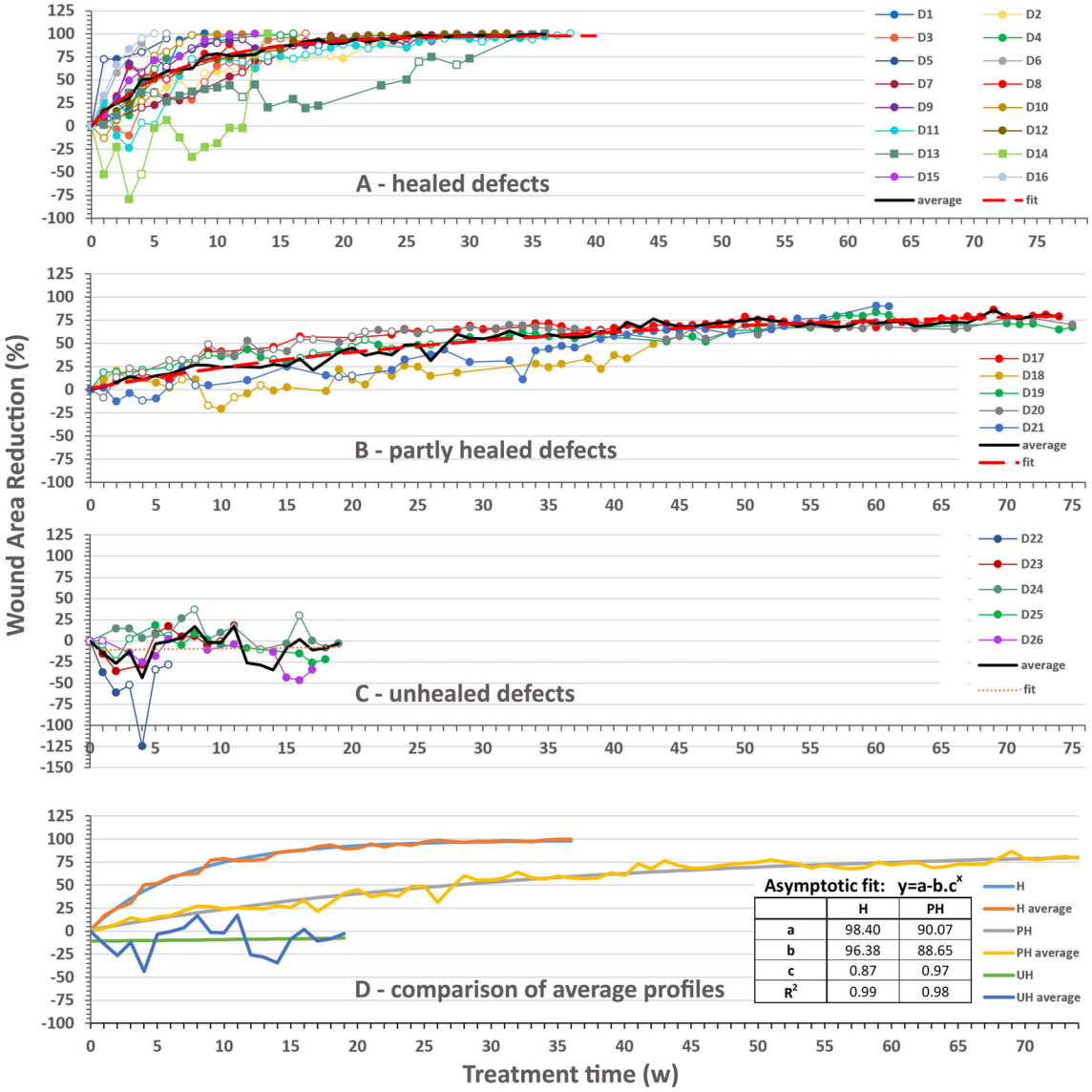

FIGURE 2 Wound closure evolution. WAR progress for healed A, partly healed B, and unhealed C, defects. D, comparison of the averages of the three groups together with fitted asymptotic functions and their parameters for H and PH and correlation coefficients (R^2). For A, B, and C, the closed and open markers reflect visits with or without AM application, respectively

individual defects are presented in Table 2. We have recorded the WAR profile for each defect and determined the average healing profiles separately for each group (H, PH, UH) (Figure 2). From the average WAR curve for healed defects (H) and its fit (regression coefficient $R^2 = 0.987$) (Figure 2A, D), we established that on average 50% of healing was achieved in 5 weeks of treatment and 70% in 10 weeks. All healed defects reached at least 50% of closure within the first 10 weeks of treatment (Figure 2A) with two exceptions: The defects D13, D14 (lines with square markers in Figure 2A) exhibited a profile significantly diverging from the rest of the defects in the group. They were characterised by delayed onset (12 and 17 weeks, respectively) of the AM stimulated healing and partial initial worsening. After this lag period, during which the healing profiles resembled those of the PH group, both defects started to progress and reached complete closure. The most prolonged healing in the H group lasted 44 weeks (defects D8 and D9).

Five defects (D17 to D21) healed partially, and despite the prolonged care period (up to 78 weeks) these defects progressed slowly without reaching the complete closure (Figure 1B, Figure 2B). The average WAR of these defects followed a different curve compared with the H group's characteristics, and approximately 27 weeks were necessary to reach 50% of closure (average). The average WAR value was 71% at the end of treatment. The course of the fitted curve is significantly flatter compared with the H group and predicts the maximum reachable WAR of approximately 90% (a = 88, Figure 2D).

Four patients (five defects, D22 to D26) did not respond to the AM treatment despite intense care, Figure 1C, Figure 2C. Their WAR values oscillated around 0, meaning that their size randomly changed around the baseline. The very approximate linear fit shows virtually no effect of the treatment on the wound size.

The degree of pain in all patients decreased independently of the healing progress from an average of 3.25 before the first AM application to 1.95, 1.22, and 0.47 after the first, fifth, and the tenth week of AM treatment, respectively, on a scale from 0 (no pain) to 10 (the worst pain) (Figure 3). No difference in pain relief was found between healed and unhealed patients.

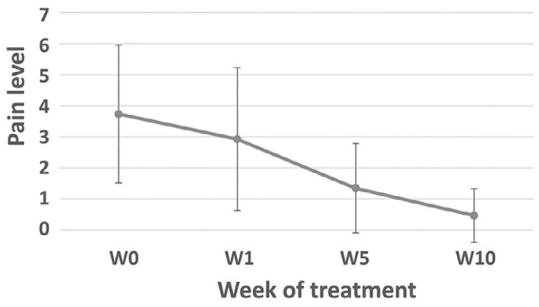

FIGURE 3 Pain level evolution during the AM treatment. Average value ± SD from all patients on a scale from 0 (no pain) to 10 (the worst pain) at week (W) 0, 1, 5, and 10 of treatment

8 | DISCUSSION

When AM is used for the treatment of NHW, several factors may affect the outcome: the wound aetiology, baseline size, period from onset to treatment start, type of AM used (cryo-preserved, dried, lyophilised, etc.), frequency of AM application, treatment period, and the individual sensitivity to the AM effect, which can be directly related to the general health status of the subject's organism, including age, and BMI and comorbidities of the subjects. In this multicentric study, we assessed the effect of cryo-preserved AM application on the healing of NHW. Apart from evaluating the general healing effect, one of the aims of the study was to understand if there is a way to predict the outcome of the AM stimulated healing process already at the early stages of the treatment. Although the logistics (long-term storage and the delivery in frozen state) related to the use of such material is more complex than that of, for example, dried or lyophilised tissues, cryo-preserved AM should preserve the majority of the growth factors and other molecules affecting the healing process in their active form and, therefore, promote the treatment efficiency.[7,27-29] Some studies, however, indicate that the quality of lyophilised AM levels that of cryo-preserved AM in terms of preservation of active molecular components[30] and efficiency in NHW treatment.[18]

The observed healing rate of 62% in our study is concordant with values reported in similar studies. In a multicentre, controlled, randomised, blinded, clinical trial, Lavery et al observed 62% successful healing rate (100% reepithelialisation at 12 weeks) for cryo-preserved AM compared with 21.3% success rate using SOC.[31] In a retrospective study evaluating 350 NHW from WoundExpert electronic health records, the rate of closure using cryo-preserved AM was found to be 59.4%.[32] Farivar and colleagues,[33] in a trial limited to 12 weeks and venous leg ulcers, reached 53% healing with a comparable set of NHW when using cryo-preserved AM and the average healing time of 10.9 weeks, which is markedly shorter compared with 25.5 weeks in our study. It should be noted, however, that the average NHW duration period before AM treatment in our patients was 88 weeks, which is considerably higher than in Farivar's study and other comparable studies using cryo-preserved AM.[24,31,34] Interestingly, in Ref. 33, 57% of unhealed defects showed WAR >50%, which is very similar to our 50% (five defects) representing the PH group. Another

multicentre trial reported a success rate of 48.4% (100% reepithelialisation at 8 weeks) for viable cryo-preserved placental membrane.[34]

In general, the reported in literature rate of complete wound closure using cryo-preserved AM are quite dispersed from 20%[23] through 46%,[31] 53%,[33] 84%[35] to 100%.[24] However, some studies were realised with limited AM application period reducing, thus, the potential healing completion had the treatment been continued. Time to complete closure appears to be also variable, ranging from 6 to 56 weeks.[23,24] Therefore, the extended healing period for some defects in the present work is in the range of values reported previously.

Some works report the healing efficiency's dependence on the baseline size.[32,34,36] In our study, although the average baseline size is superior in the UH group compared with H group, we could not prove its statistical significance. The average wound size before starting AM therapy (15.4 cm^2) was larger in our study than in other studies using cryo-preserved AM.[24,31,34,35]

The frequency of AM or AM-based derivatives application used in similar studies varies from frequent (2 to 3 days[37]) to scarce.[23] We set the standard application period to weekly at the beginning of treatment. In later phases (generally after 4 to 8 weeks), the AM application frequency was changed to bi-weekly and then modulated according to the wound reaction to the treatment and healing evolution.

AM treatment cost is rather high. Thus, the question at which state its application is adequate and what are the indications suggesting its efficiency is important. Existing data show that WAR of 50% after 4 weeks of SOC is a strong indicator of appropriate healing for diabetic foot ulcers.[38] WAR change in percent was reported as an indicator of a good prognosis for venous leg ulcers.[26,39] Failure to reach such rates by SOC suggests the need for initiating alternative and/or advanced treatments.[40]

When AM application is undertaken, an indicator of the probability of complete healing would be a valuable tool for deciding whether the treatment should be continued for an extended period or not. Analysis of our results allowed us to separate the wounds into three categories with different sensitivity to the AM treatment—H, PH, and UH. The wounds in the H group achieved, on average, 70% closure after 10 weeks and 50% after 5 weeks, which is similar to the values reported elsewhere.[24] All wounds in this group attained 50% closure in 10 weeks; therefore, it would seem adequate to establish these values as predictors for successful treatment. However, this criterion may exclude the cases that would have finally benefited from the AM application had the treatment not been aborted (D13, 14). The average WAR progress for NHW in PH and UH groups is different. The wounds completely insensitive to the AM application (UH group) are readily recognisable at the early stages of treatment (10 weeks at maximum) by the flat average progression line. To distinct NHW with the potential to heal completely (H) or to be only partially reduced over prolonged treatment (PH) is, however, more complicated. We have characterised the healing progress of these two groups by parameters of the fitted asymptotic function. The determined 'a' parameter defines the maximum reachable value of wound closure and thus can be used to predict the extent to which the wound would heal. Compared with the H group, the PH group shows significantly slower evolution with the indicated limit of only ~90% ('a' value), suggesting that these wounds will never reach complete healing when using this type of treatment, and probably it is of low interest to continue with rather expensive AM application. Instead, the treatment strategy should be reconsidered. Unfortunately, the data spread is wide, and therefore such distinction at the level of an individual patient can hardly be applied with high certitude before the weeks 12 to 13 of treatment. Interestingly, this value coincides with the time limit of most studies evaluating the effect of skin substitutes (for a complete overview, see Ref. 26.

Our results also show that the repeated and persistent application can eventually trigger the healing process even in defects not responding immediately to the AM treatment and not fitting into the above-stated values for H-type wounds. That was indeed the case of defects D13 and D14, which for an extended period exhibited only a minimal response to the treatment and reached the 50% closure only after 25 and 12 weeks, respectively, but finally healed completely. The important feature of AM is, besides healing acceleration and stimulation, its analgesic effect; pain relief has been perceived after first AM applications by all patients independently of wound healing. In any case, the AM application in NHW healing will always require a highly individual approach and should reflect previous wound treatment/healing history and the patient's general health condition.

9 | CONCLUSION

From our results, we conclude that cryo-preserved AM represents a safe and useful treatment with a strongly beneficial effect for managing NHWs. In our study, the AM application led to the complete healing of 62% of wounds. We have shown that it was possible to separate the NHW into three distinct groups with different healing characteristics (H, PH, U) according to their WAR profiles. This can then be used as a guideline for the prediction of the eventual outcome of the treatment.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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